

**SIGNAL TRANSDUCTION PATHWAYS  
MEDIATING THE EFFECTS  
OF  
PROGESTERONE-INDUCED BLOCKING FACTOR**

**Ph. D. Thesis**

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## I. LIST OF ABBREVIATIONS

7-AAD	7-aminoactinomycin D
AA	arachidonic acid
aPKC	atypical protein kinase C
ASA	acetylsalicylic acid
BSA	bovine serum albumin
CBA	cytometric bead array
COX	cyclooxygenase
cPKC	conventional protein kinase C
DTT	dithiothreitol
ELISA	enzyme linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
FCS	fetal calf serum
FITC	fluorescein-isothiocyanate
GPI	glycosylphosphatidylinositol
HRPO	horse radish peroxidase
IL	interleukin
IL-12R	IL-12 receptor
IL-13R $\alpha$ 1	IL-13 receptor $\alpha$ 1
IL-4R	IL-4 receptor
INF	interferon
Jak	janus kinase
LPS	lipopolysaccharide
NK	natural killer
nPKC	novel protein kinase C
NSAID	non-steriod anti-inflammatory drug
ODP	O-phenylenediamine
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PG	prostaglandin
PHA	phytohemagglutinin
PIBF	progesterone-induced blocking factor
PIBFR	progesterone-induced blocking factor receptor
PI-PLC	phosphatidylinositol-specific phospholipase C
PKC	protein kinase C
PLA <sub>2</sub>	phospholipase A2
PLC	phospholipase C
PR	progesterone receptor
Q	quinacrine
RNAi	RNA interference
SCR	non-genomic sequence oligonucleotide/scrambled
SH2	Src homology-2 domain
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
TBS	tris-buffered saline
TCR	T cell receptor
Th	T helper
TNF $\alpha$	tumor necrosis factor $\alpha$

## **II. PROLOGUE**

The fetus can be considered an allograft, since it inherits fifty per cent of its antigens from the father. Reproductive immunology deals with the complex regulatory mechanism, which enables the genetically and immunologically incompatible fetus not only to survive, but to develop within the potentially hostile maternal immunological environment for several months till delivery. Several concepts have been proposed to explain, how the fetus avoids being rejected by the maternal immune system, but the complex immuno-endocrine mechanism accounting for fetal survival is still not completely understood.

Autoimmune diseases, infections, malignant tumours show different symptomatology and altered epidemiology during pregnancy. In order to protect the mother from infectious diseases, malignancies and prevent at the same time unwanted immunological attack to the fetus - the immune system ensures a normal balance characterized by a temporary suppression of cell-mediated immune effector functions and a predominance of humoral immune response.

Successful pregnancy is a natural model of an immune regulation in a graft-host relationship, where the homeostasis is created by a bidirectional interaction between the immune and the endocrine systems.

### **III. INTRODUCTION**

#### **1. Progesterone dependent immunomodulation**

Progesterone is essential for the maintenance of pregnancy in most mammalian species. At the beginning of pregnancy it is produced by the corpus luteum and later by the placenta. Progesterone modifies the activity of proteolytic enzymes, blocks the uterine collagenase activity (1) and inhibits the contractions of myometrial smooth muscle (2). High concentrations of progesterone prolong the survival of xenogenic and allogenic grafts (3, 4) and affects different stages of the immune response (5, 6). Progesterone was shown to inhibit in physiological concentrations natural cytotoxic activity of pregnancy lymphocytes, whereas lymphocytes of non-pregnant individuals did not respond to the same concentrations (7). Increased progesterone sensitivity of pregnancy lymphocytes is due to their higher progesterone binding capacity (8).

Peripheral lymphocytes of pregnant women express specific progesterone receptors (PRs) (9, 10, 11), which might account for the previously mentioned higher progesterone sensitivity of the cells. Unlike the classical PRs, lymphocyte PRs are up regulated by activation of the cells, and their regulation is independent from the hormonal environment (12). Therefore, these PRs are absent from nonpregnancy lymphocytes (9), but appear as early as the tenth day of gestation and disappear during term and preterm labour, as well as during spontaneous abortion (13).

A study by Chiu et al in 1996. showed, that an effective immunotherapy with paternal lymphocytes for unexplained recurrent spontaneous abortion resulted in increased PR expression on maternal lymphocytes. Since the presence of lymphocyte PRs is related to normal pregnancy, but their disappearance is associated with pregnancy termination, it

is conceivable that the lack of lymphocytic PRs during pregnancy might have functional consequences.

### **1.1 Progesterone-Induced Blocking Factor**

The biological effects of progesterone are manifested via a mediator protein named the Progesterone-Induced Blocking Factor (PIBF), which is released by lymphocytes of healthy pregnant women in the presence of progesterone (14). PIBF is coded on locus 13q21-q22, which has been implicated as a common site for somatic deletions in a variety of malignant tumors (15) and has recently been identified in rapidly proliferating and undifferentiated cells, as well as in malignant tumors (16).

When PIBF was originally discovered, the biological functions were identified with a 34-36 kDa-secreted protein (14). Data from our laboratory showed that the cDNA of PIBF encodes a 90-kDa molecule (17). The full-length PIBF is associated with the centrosome (18), and the secretion of different protein forms that are synthesized due to alternative splicing (16), begins upon activation of the cell.

PIBF has pleiotropic biological properties:

- It interferes with arachidonic acid (AA) metabolism.
- PIBF alters the cytokine profile. Successful pregnancy is characterized by a T helper (Th) 2 dominant cytokine production of peripheral lymphocytes (19) and a Th2 shifted cytokine pattern is thought to be important for a normal pregnancy. PIBF induces a significant increase in IL-10, IL-3 and IL-4 production by activated murine spleen cells and inhibits the IL-12 synthesis of human pregnancy lymphocytes (20). The anti-

PIBF treatment of pregnant mice reduced splenic IL-10 production and resulted in increased NK activity together with high resorption rate (21).

- It blocks Natural Killer (NK) cell- mediated cell lysis, and depresses the mixed lymphocyte reaction.
- PIBF exerts anti-abortive effect in mice by blocking NK activity and modifying cytokine production (22, 23). The neutralization of endogenous PIBF or the blocking of progesterone binding sites by PR antagonists causes abortion in mice (24, 25, 26). Anti-PIBF induced pregnancy termination is associated with an increased splenic NK activity (8) in mice. Increased resorption rates in anti-PIBF treated mice are corrected by the treatment of pregnant animals with anti-NK antibody (8). These data suggest that – at least in mice – PIBF is indispensable for the success of pregnancy and that its pregnancy-protective effect is manifested via inhibiting NK activity.

Although in humans there is no direct proof for a key role of PIBF in the maintenance of pregnancy, PIBF concentrations in sera of healthy pregnant women are higher than in those of non-pregnant individuals or pregnant women with symptoms of threatened abortion (18). PIBF expression of the lymphocytes shows an inverse correlation with NK activity and the rate of PIBF positive lymphocytes is related to the outcome of pregnancy (14, 22). This experimental evidence implicates PIBF as a crucial factor in the immunomodulation during normal pregnancy.

The N-terminal 48-kDa part of recombinant PIBF inhibits NK activity to the same extent as the secreted lymphocyte PIBF does. This effect is neutralized by antibodies specific for the secreted PIBF. Similarly to the secreted PIBF, the recombinant molecule increases IL-10 production and inhibits IL-12 production by peripheral lymphocytes, both in a concentration dependent manner. These data suggest that the biologically active site,



responsible for modulating NK activity, lies within the 48-kDa N-terminal part of the molecule (17).

## **1.2 Immunological effects of prostaglandins during pregnancy**

Earlier observations revealed a relationship between prostaglandin (PG) levels, progesterone binding capacity and cytotoxic activity of lymphocytes (27).

PIBF maintains low NK activity via multiple mechanisms. PIBF inhibits NK cell cytotoxicity by increasing IL-10, and decreasing IL-12 production (28) and it inhibits degranulation and perforin liberation from NK cells and peripheral lymphocytes (29). PG output is modulated by pro-inflammatory cytokines.  $\text{TNF}\alpha$ , IL-6 and IL-1 $\beta$  activate cells to increase PG synthesis, but the stimulatory effect of pro-inflammatory cytokines is down regulated by progesterone (30).

Progesterone / PG imbalance (low serum concentration of progesterone with high serum concentration of PGs) is related to pregnancy termination (31). There is an inverse relationship between placental progesterone levels and PG dehydrogenase activity, implying that progesterone / PG balance might be regulated by inhibition of PG dehydrogenase by progesterone in the placenta (32). PGE<sub>2</sub> is thought to have a mainly inhibitory effect on lymphocytes, whilst PGF<sub>2</sub> $\alpha$  stimulates lymphocyte activity (33, 34). According to Kuell et al. the characteristic changes in PGE<sub>2</sub> / PGF<sub>2</sub> $\alpha$  ratio are strongly dependent on the alterations of the progesterone / estrogen ratio during pregnancy (35).

The immediate precursor of PGs is AA. AA possesses a marked NK activating effect *in vitro* (36). AA is synthesized from phospholipids in the cell membrane by Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) or by a two step reaction catalyzed by Phospholipase C (PLC) and diacylglycerol lipase. The formation of PG is catalyzed by the cyclooxygenase (COX)

enzyme, which is located in the endoplasmatic reticulum. The transformation of AA into PGs involves two reactions by COX. The cyclization is the first step and it is followed by a peroxydase reaction. There are two main forms of COX (recent data suggest a third type 37). COX1 is expressed constitutively and it plays a critical role in maintaining homeostasis, while COX2 is induced by pro-inflammatory stimuli. The inhibition of COX2 is responsible for the anti-inflammatory effect of non-steroid anti-inflammatory drugs (NSAID), whereas COX1 inhibition accounts primarily for the side effects.

There is a well established relationship between intrauterine infections and premature delivery (38, 39, 40). Bacterial endotoxins, which show elevated level in the amniotic fluid during intrauterine inflammation (41) by acting on COX2, stimulate PG synthesis. High PG levels induce myometrial contractions and at the same time exert an immunological effect (42, 43, 44). Increased concentrations of Th1 type cytokines are present during intraamniotic infection (45). Lipopolysaccharide (LPS) treatment in pregnant mice results in an enhanced PG synthesis and increased NK activity (46).

The impact of immunological effects of the COX inhibitor: Acetylsalicylic Acid (ASA) on the outcome of pregnancy were investigated by Szekeres et al. (33). ASA treatment of women showing symptoms of threatened abortion resulted in a significant reduction in cytotoxic activity and increased the progesterone binding capacity of lymphocytes remarkably. The long term administration of ASA resulted in a significantly lower abortion rate compared to „risk patients” receiving only traditional beta mimetic anti-abortion treatment (33).

## **2. The importance of Jak/STAT and PKC/Ca<sup>++</sup> pathways in cytokine production**

Signal transduction is a vague sort of blanket term, for what goes on inside a cell after a hormone, growth factor, neurotransmitter, adhesion molecule, substrate molecule or other signal has hit the cell membrane. There are many signalling mechanisms by which cells respond in a specific way to a wide variety of extracellular regulatory substances.

### **2.1 STAT and SOCS signalling**

The Signal Transducer and Activator of Transcription (STAT) proteins were identified in the last decade as transcription factors critical in mediating virtually all cytokine driven signalling (34). They have attracted attention because of their special mode of activation and the diversity of the resulting biological effects. STATs provide a direct link between the cytokine receptors and cytokine induced gene transcription. STATs are latent cytoplasmic proteins that are promptly activated by tyrosine phosphorylation by the cytokine receptor associated Janus kinases (Jaks). STAT phosphorylation allows the dimerization of individual STAT proteins via their SH2 domains, the dimers are then capable of migrating directly to the nucleus, where they can bind DNA and directly activate cytokine responsive gene transcription (35). STATs were first identified as a unique family of DNA-binding proteins in 1993 (36) playing a role in interferon (INF)-regulated gene expression.

Following the cloning of STAT1 and STAT2, it became obvious that STAT-like activities were elicited by various cytokines. This prompted efforts that resulted in the identification of five additional mammalian STAT genes. STAT3 was cloned as an IL-6

activated factor by homology to STAT1. STAT4 was cloned by homology approaches; STAT5 was cloned as a prolactin-activated transcription factor from sheep. STAT6 was cloned as an IL-4-activated DNA binding factor.

STATs share several conserved structural and functional domains. The most interesting and conserved domain is a potential phosphotyrosine-binding, SRC homology SH2 domain (SH2). It is critical for the recruitment of STATs to activated receptor complexes and it is required for the interaction with the Jaks, which phosphorylate the STATs. Finally the SH2 domain is necessary for STAT dimerization and the associated ability to bind DNA (37).

IL-4 and IL-13 are the primary activators of STAT6. In primary B lymphocytes both Ig and CD40 cross-linking can induce STAT6 tyrosine phosphorylation. In the case of Ig cross-linking, there appears to be additional dependence on Protein Kinase C (PKC) to mediate this signal. The primary activation of STAT6 occurs via the ligation of the IL-4 receptor (IL-4R).

The IL-4R is a heterodimer cytokine receptor system consisting of the signalling high-affinity  $\alpha$  chain and a trans-activating low-affinity chain that can be either the common  $\gamma$  chain or the IL-13 receptor  $\alpha 1$  (IL-13R $\alpha 1$ ) chain (38). The extracellular domain of the IL-4R $\alpha$  chain, called IL-4-binding protein, provides nearly the complete ligand-binding affinity of the whole receptor (39). IL-4R $\alpha$  and  $\gamma$  chain associate with the Janus kinases, Jak1 and Jak3 respectively (40). Ligand-induced heterodimerization of IL-4R $\alpha$  and  $\gamma$  chain in lymphoid cells induces tyrosine phosphorylation of the IL-4R $\alpha$  chain and activation of Jak1 and Jak3 (41). Phosphorylated tyrosine residues of IL-4R $\alpha$  chain serve as anchor for the SH2 domain of STAT6, which in turn gets phosphorylated (42). Phosphorylated STAT6 forms homodimers that translocate to the nucleus, where they bind

to DNA sequences TTCNNN(N)GAA in the promoter region of IL-4 responsive genes (43).

STAT6-deficient T helper cells are unable to differentiate into Th2 cells *in vitro* and *in vivo* (35). STAT6-deficient mice are markedly impaired in their ability to respond to IL-4. The expression of genes, known to be regulated by IL-4, such as CD23, IL-4R $\alpha$  and MHC class II molecules, fails to increase following the IL-4 stimulation of STAT6-deficient lymphocytes. STAT6-deficient mice do not generate an IgE response, following helminthic infection or immunization with the polyclonal stimulus anti-IgD. Finally the differentiation of Th2 cells by STAT6-deficient lymphocytes was found to be markedly impaired (44).

STAT4 plays an important role in Th1 cell function and development. STAT4 is mainly phosphorylated by engagement of the IL-12 receptor (IL-12R). The IL-12R is composed of two chains, termed IL-12R $\beta$ 1 and IL-12R $\beta$ 2. Ligand binding results in heterodimer formation and activation of the receptor associated Janus kinases, Jak2 and Tyk2. Having been phosphorylated by these tyrosine kinases, STAT4 forms homodimers via its SH2 domain and translocates into the nucleus, where it recognizes traditional N3 STAT target sequences in IL-12 responsive genes. Although IL-12 appears to be the predominant activator of STAT4, in human cells STAT4 can also be phosphorylated in response to INF $\gamma$  stimulation (45).

STAT4-deficient mice show impaired IL-12-induced responses. IL-12-induced INF $\gamma$  production, cellular proliferation and NK cytotoxicity are all abrogated in the absence of STAT4. The differentiation of INF $\gamma$  producing Th1 cells is markedly impaired in STAT4-KO mice.

IL-12/STAT4 and IL-4/STAT6 pathways are under negative feedback regulation by Suppressor of Cytokine Signalling (SOCS) Proteins (46, 47). The SOCS family of

proteins is composed of eight members characterized by the presence of Src homology-2 domain (SH2) and a C-terminal conserved domain called the SOCS box. The inhibitory effects derive from direct interaction of SOCS SH2 domains with cytokine receptors and/or Jaks, leading to the recruitment of SOCS proteins to the signalling complex, to the inhibition of STAT binding to tyrosine-phosphorylated cytokine receptors and to the suppression of the catalytic activities of Jaks (48, 49, 50).

SOCS1 and SOCS3 genes are differentially expressed in Th1 and Th2 cells. CD4<sup>+</sup> naive T cells constitutively express low levels of both SOCS1 and SOCS3 mRNAs, and differentiation into Th1 or Th2 phenotype is accompanied by preferential expression of distinct SOCS mRNA transcripts and proteins (51). Egwuagu et al. previously reported that Th2 cells, where IL-4/STAT6 signalling is activated and IL-12-induced STAT4 phosphorylation is inhibited, contain 23-fold higher levels of SOCS3 than Th1 cells, in which 5-fold higher levels of SOCS1 are measured (51). SOCS3 is induced by IL-4 signal, and inhibits IL-12 induced STAT4 activation by binding to the IL-12R. The SOCS3 SH2 domain and the docking site (shared with STAT4) in IL-12R $\beta$ 2, Tyr-800, are involved in this interaction (52).

The critical and exquisitely specific roles of STAT4 and STAT6, as well as of SOCS1 and SOCS3 factors in T helper cell differentiation suggest that these proteins would be ideal therapeutic targets for immune modulation to skew the immune response toward a desirable outcome.

## **2.2 PKC and Ca<sup>++</sup> signalling**

The PKC pathway represents a major signal transduction system that is activated following the ligand-stimulation of receptors by hormones, neurotransmitters and growth

factors. PKC plays a critical role in the regulation of differentiation and proliferation in many cell types and in the response to diverse stimuli.

T cells express multiple isotypes of PKC and though it is accepted that PKCs play a role in T cell activation, little is known about the function of individual PKC isotypes. At least 11 isoenzymes have been described and classified into 3 subfamilies:  $\text{Ca}^{++}$  dependent or conventional protein kinase C (cPKC) isoforms;  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\text{Ca}^{++}$  independent, novel protein kinase C (nPKC) isoforms;  $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ,  $\mu$  or atypical phospholipase- and  $\text{Ca}^{++}$  independent protein kinase C (aPKC) isoforms  $\zeta$ ,  $\iota$ ,  $\lambda$  (53).

T cells express multiple isotypes of PKC and though it is accepted that PKCs play a role in T cell activation, little is known about the function of individual PKC isotypes.  $\text{Ca}^{++}$  independent PKC isoforms were involved in a PKC-induced Th2 differentiation (54, 55, 56) in most studies. The loss of PKC $\zeta$  leads to impairment in the secretion of Th2 cytokines due to the inability of the PKC $\zeta^{-/-}$  CD4 $^{+}$  T cells to differentiate adequately along the Th2 lineage, furthermore based on the currently available data, PKC $\zeta$  is critical for IL-4 signalling and Th2 differentiation (57). According to the latest reports, PKC $\zeta$  levels are increased during Th2 differentiation of CD4 $^{+}$  T cells. The lack of PKC $\zeta$  impairs the secretion of Th2 cytokines *in vitro* and *in vivo*, as well as Jak1 activation and tyrosine phosphorylation. The nuclear translocation of STAT6 is inhibited in PKC $\zeta^{-/-}$  adult mice, they are unable to mount an optional immune response. The dramatically inhibited phosphorylation of STAT6 was also detected in PKC $\zeta^{-/-}$  cells and the exogenous addition of IL-4 could not bypass this blockade (58).

PKC $\theta$  is critical for the development of *in vivo* Th2- but not Th1 cell responses. PKC $\theta$ -deficient T cells exhibit impaired IL-4, but normal INF $\gamma$  production (56). PKC $\theta^{-/-}$  mice fail to develop Th2 cell-dependent allergic airway inflammation. The lack of IL-4 production is consistent with the reduced level of IgE detected in *in vivo* models (56).

Among others, the development of naive T cells into type 1 (Th1) or type 2 (Th2) effector cells is thought to be under the control of cytokines. IL-12 and IL-4 are widely accepted to be the major factors inducing T cells to develop into type 1 or type 2 direction (59). When IL-12 and IL-4 are present, murine and human T cell differentiation is regulated by the balance of PKC and  $\text{Ca}^{++}$  signalling within T cells (54). Th2 clones show reduced  $\text{Ca}^{++}$  flux after activation compared with Th1 clones (60). The high levels of PKC activity combined with low  $\text{Ca}^{++}$  signals favour Th2 development, while the predominance of  $\text{Ca}^{++}$  signalling with low PKC activity favours Th1 development (54). Signals downstream of PKC and calcineurin directly result in preferential type 1 or type 2 cytokine gene expressions, via activation of transcription factors associated with Th2 cells (61, 62).

These mechanisms, whereby the balance of intracellular signals is induced via T cell receptor (TCR) stimulation, have a primary influence on the decision - a developing cell makes between the type 1 or type 2 pathways.



#### IV. AIMS OF THE STUDY AND RESULTS

It is well established that pregnancy is characterized by an altered cytokine balance induced by progesterone-dependent immunomodulation. Progesterone exerts its biological effects inducing Th2 shift via a regulator protein, named PIBF. The aim of this study was to investigate; the pathways that might be involved in mediating the cytokine effects.

##### 1. Phospholipase A 2 is involved in progesterone-dependent immunoregulation.

PIBF inhibits NK cytotoxic activity *in vitro* (63) and exerts an anti-abortion effect *in vivo*. PIBF also affects the cytokine secretion of peripheral lymphocytes by increasing IL-10 and decreasing IL-12 production (28).

Earlier studies revealed a relationship between PGF2 $\alpha$  levels, progesterone binding capacity and cytotoxic activity of the lymphocytes (27). Par et al. observed a significantly increased IL-12 production in anti-PIBF treated pregnancy lymphocytes, which was corrected by indomethacin treatment (64).

It is likely that PIBF acts before the level of cyclooxygenase and lipoxygenase enzymes, as the blocking effect on cytotoxic activity is voided in the presence of exogenous AA (65). Therefore, we investigated the effect of PIBF neutralizing antibody and simultaneous phospholipase A<sub>2</sub> (PLA<sub>2</sub>) inhibitor: quinacrine (Q) treatment upon IL-12 production.

LPS is known to selectively stimulate PG synthesis and to enhance cytotoxicity (66). In our hands, LPS treatment increased the percentage of IL-12 positive cells significantly, and the specific cyclooxygenase inhibitor, indomethacin corrected this effect (67). Furthermore, Q decreased LPS induced IL-12 expression significantly, in a

concentration dependent manner, reaching a complete block at a concentration of 10 $\mu$ M. The treatment of lymphocytes with PIBF neutralizing antibody results in a significant increase of IL-12 positive mononuclear cells. We found, that IL-12 production was stimulated significantly, when endogenously produced PIBF was neutralized by anti-PIBF antibody, and the combined treatment with PLA<sub>2</sub> inhibitor corrected anti-PIBF induced increased IL-12 production. The above data suggest that PIBF affects AA release. The subsequent block of PG synthesis reduces IL-12 production and results in a lowered NK activity, which favours a normal pregnancy outcome (Paper 1).

## **2. The effects of PIBF on STAT and SOCS signalling.**

PIBF induces a Th2 biased cytokine production *in vitro* (28, 68) and *in vivo* (69). Activated lymphocytes produce increased amounts of Th2 type cytokines: IL-3, IL-4 and IL-10 in the presence of PIBF (28). The neutralization of endogenous PIBF activity results in an altered cytokine production and pregnancy termination in mice (70).

Since PIBF exerts its biological effects by inducing a Th2 bias, and the STAT transcription factors mediate cytokine driven signalling, we investigated the effects of PIBF on the STAT6/STAT4 signal transduction pathways.

STAT proteins are latent in the cytoplasm. Following the activation of the cell, they become phosphorylated and form homo- or heterodimers. These dimers enter the nucleus, where they bind to specific DNA elements, and in cooperation with other transcriptional co-activators, or transcription factors, initiate increased transcription (71).

To test the effect of PIBF on STAT6 phosphorylation the cytoplasmic fractions of PIBF-treated or untreated peripheral human lymphocytes were separated on SDS-PAGE, blotted to nitrocellulose membranes and reacted with anti-phospho-STAT6 antibodies.

Controls included isotype controls, as well as the lysate of *E. coli* that had undergone the same purification procedure as the recombinant PIBF. Similarly to IL-4, PIBF induced phosphorylation of STAT6, and the effect of PIBF was counteracted by neutralizing anti-PIBF IgG (Fig.1).

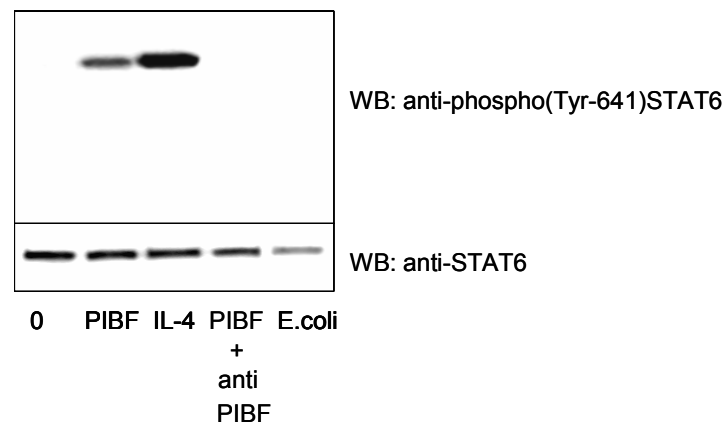


Fig.1 The effect of PIBF on STAT6 phosphorylation

To clarify whether PIBF induces nuclear translocation of phosphorylated STAT6 dimers, nuclear extracts prepared from cells treated with 48-kDa N-terminal recombinant human PIBF, and those of IL-4 treated, as well as untreated lymphocytes were hybridized with radioactive labeled STAT6 binding specific oligonucleotide probes 5' TCGACTTCCCAAGAACAGCA 3' and their reverse complementary pairs. The samples were separated by non-denaturing PAGE and the bands were detected by autoradiography. PIBF induced the nuclear translocation of STAT6 proteins in lymphocytes. Supershift assay was performed to determine the specificity of the reaction. Anti-STAT6 IgG reacting with STAT6 results in the formation of STAT6/radioactive probe/antibody complexes, which move slower in the gel, than the smaller STAT6/probe complexes. PIBF treatment resulted in the nuclear translocation of STAT6. In the presence of an anti-STAT6 antibody, a supershifted complex appeared in the extract of

PIBF-treated lymphocytes. This band was not detectable, when an irrelevant antibody (anti-NF- $\kappa$ B) was used (Fig.2).

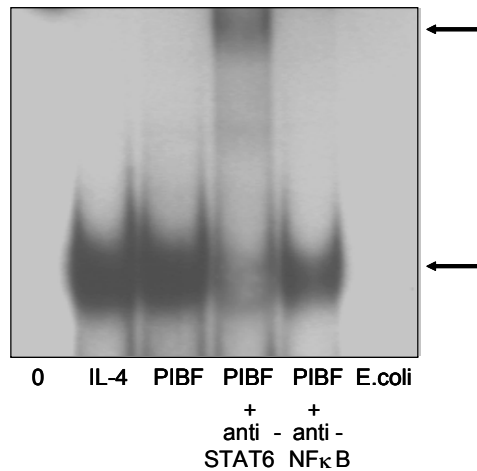


Fig.2 The nuclear translocation of STAT6 dimers induced by PIBF

Next we investigated the concentration- and the time-dependent effect of PIBF. STAT6 phosphorylation was tested on the lysates of lymphocytes incubated with different concentrations of PIBF, or with 200 ng/ml of PIBF for varying periods. Tyrosine phosphorylated STAT6 appeared, as early as 1 min after addition of IL-4, or PIBF to the cells, whereas a 24 h continuous presence of progesterone was required for the same effect. PIBF in concentrations from 10 ng to 10  $\mu$ g/ml for  $5 \times 10^7$  cells exerted a concentration-dependent effect on STAT6 phosphorylation (Fig.3 ).

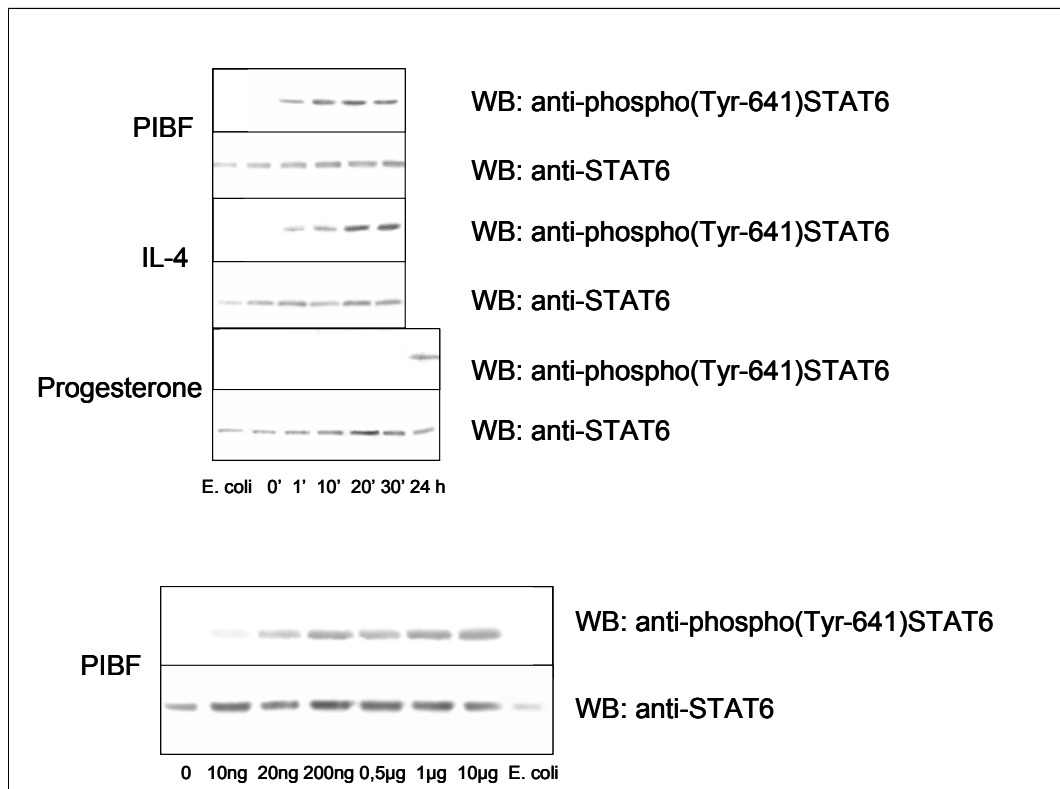


Fig.3 The concentration and time-dependent effects of PIBF on STAT6 induction

Our earlier data show that PIBF concentrations reach 100-300 ng/ml in the urine, during normal pregnancy, while serum PIBF levels of the same women are usually higher. In patients with malignant tumors PIBF levels are more variable (100 to 1000 ng/ml in the urine). Based on these data, we chose 200 ng/ml for further treatments as a concentration relevant to *in vivo* biological situations.

Multiple mechanisms maintain low NK activity during successful pregnancy. PIBF inhibits NK cell cytotoxicity by increasing IL-10 and decreasing IL-12 production (28). Studies on STAT4 knockout mice revealed that STAT4 is necessary for the generation of Th1 cells and its primal activator cytokine is the IL-12 (72). To examine whether this effect was achieved via STAT signalling, we tested the effect of PIBF on STAT4 phosphorylation in the presence of IL-12. A marked STAT4 phosphorylation in response to IL-12 was inhibited by PIBF treatment (Fig.4).

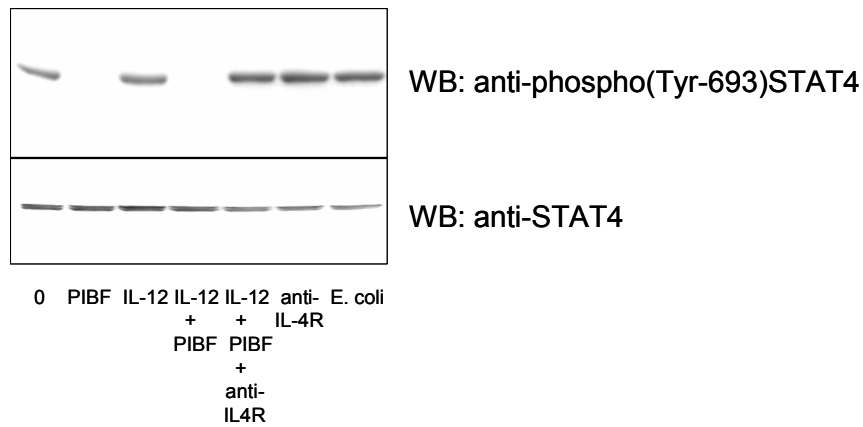


Fig.4 The effect of PIBF on STAT4 phosphorylation

IL-12/STAT4 and IL-4/STAT6 transcription are under negative control regulation by SOCSs (46, 47). Naive T cells express low levels of both SOCS1 and SOCS3 mRNAs, but their differentiation into Th1 or Th2 phenotypes is accompanied by preferential expression of distinct SOCS transcripts and proteins. Recent studies revealed, that IL-12-induced STAT4 activation is inhibited, but IL-4/STAT6 signalling is constitutively activated in Th2 cells with high levels of SOCS3, but not in Th1 cells, with high SOCS1 expression (51). IL-12 induced STAT4 inhibition is caused by the binding of SOCS3 to IL-12R subunit (52).

Next, we investigated the sensitive feedback regulation of SOCS factors on the PIBF induced STAT activation or inhibition. Similarly to IL-4, PIBF-treatment induced SOCS3 activation in the cytoplasmic fraction of lymphocytes; whereas IL-12 induced SOCS1 disappeared after PIBF or IL-4 treatment (Fig.5).

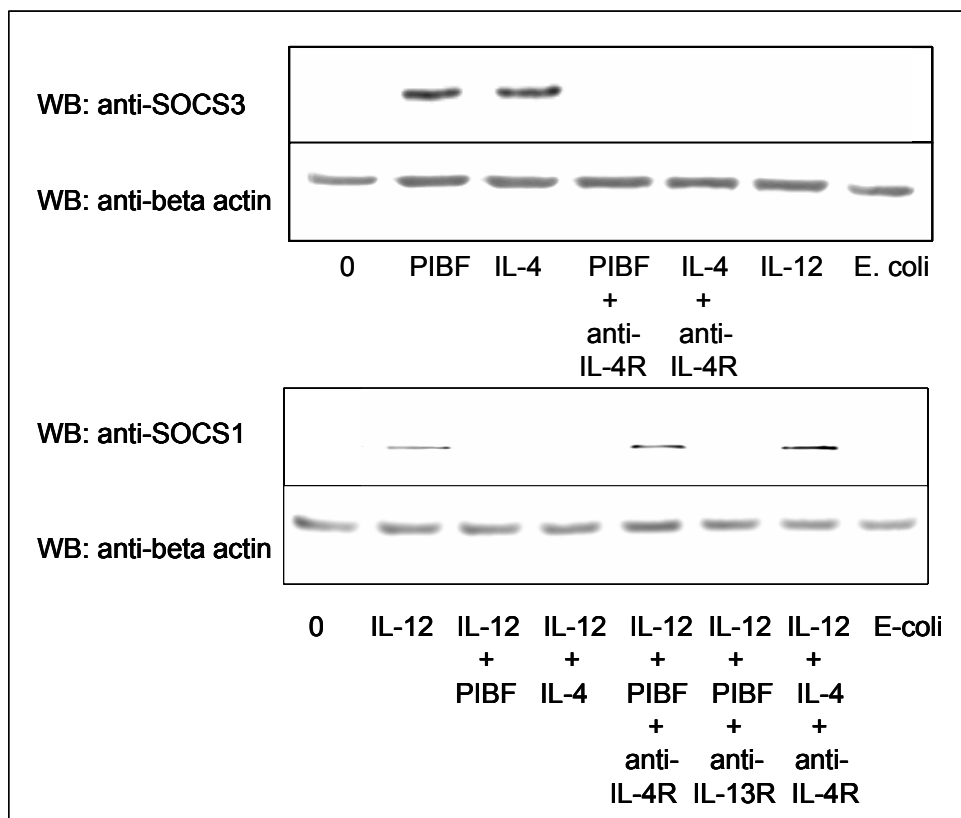


Fig.5 The effect of PIBF on SOCS1 and SOCS3 activation

In order to verify the involvement of STAT6 pathway in the cytokine effects of PIBF, STAT6 was silenced in peripheral lymphocytes with oligonucleotides interfering with STAT6 mRNA (siRNA). Cells treated with the identical nucleotides in a scrambled non-genomic order served as controls. The efficiency of STAT6 depletion was checked with the densitometry on anti-STAT6 reacted Western blots of cell lysates (Fig.6).

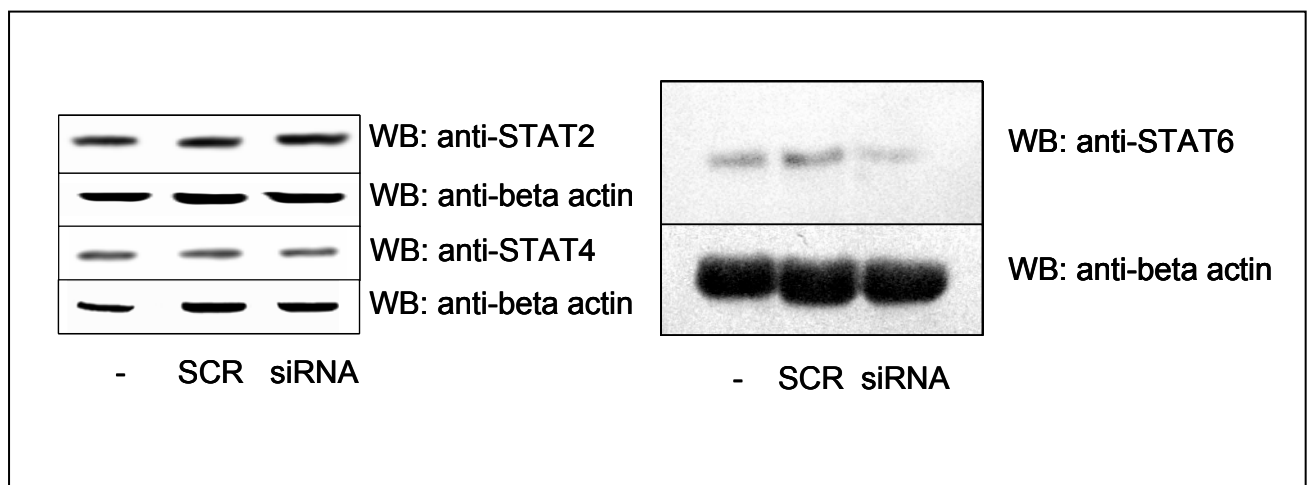


Fig. 6 The efficiency of STAT6 knock out in lymphocytes

This shows, that STAT6 was not completely silenced by this method. The relative band intensity (corresponding to STAT6) calculated by Scion Image was 43.86 in intact cells (-), 49.4 in cells treated with the non-genomic sequence oligonucleotides (SCR) and 23.72 in cells treated with the specific siRNA.

The lymphocytes were incubated with PIBF for 48h, and the IL-10, IFN $\gamma$  as well as TNF $\alpha$  concentrations of the culture media were determined with a cytometric bead assay. For controlling the effect of possible LPS contamination of recombinant PIBF, parallel, lymphocyte samples were treated with lysates of *E. coli* that had undergone the same purification procedure as the recombinant PIBF. Compared to the controls, the concentrations of both TNF $\alpha$  (Fig.7) and IFN $\gamma$  (Fig.8) were significantly increased in STAT6 deficient cells treated with 10  $\mu$ g/ml of PIBF. PIBF increased IL-10 production of intact lymphocytes in a concentration-dependent manner. Similar changes were observed in cells treated with non-genomic sequence oligonucleotides and in STAT6 deficient cells. In the latter group, PIBF-induced elevation of IL-10 production was lower compared to SCR control; however the difference was not statistically significant (Fig.9). A pitfall of the RNAi method is; that - according to recent findings - non-specific effects, that include both the up-regulation and suppression of non-targeted genes can be observed (73), which could account for elevated IL-10 levels in the supernatants of cells treated with the non-genomic sequence. Therefore SCR control might be more appropriate as a baseline than untreated cells. The efficiency of STAT6 depletion was also checked by reacting the lysates of siRNA treated cells with anti-STAT4 and anti-STAT2 antibodies. The silencing was specific for STAT6 (Fig.6).



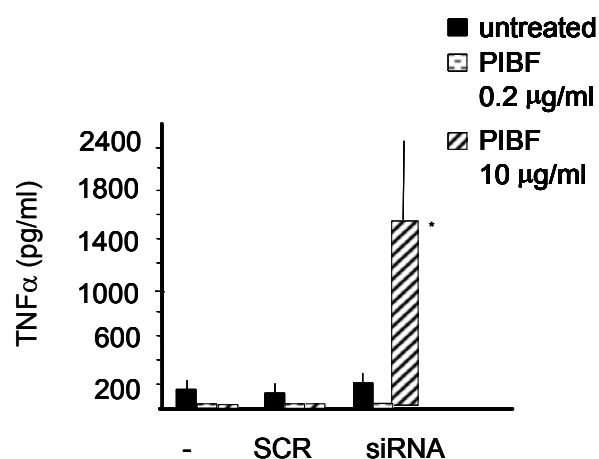


Fig.7 The production of TNF $\alpha$  induced by STAT6 deficient lymphocytes

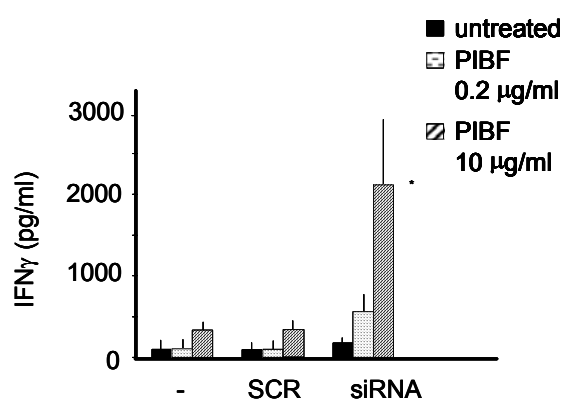


Fig.8 The production of IFN $\gamma$  induced by STAT6 deficient lymphocytes

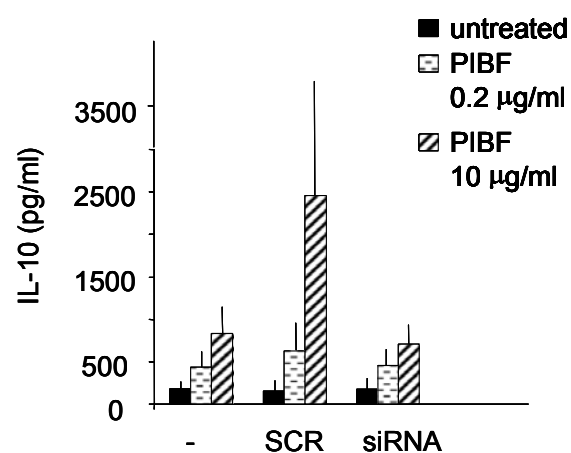


Fig.9 The production of IL-10 induced by STAT6 deficient lymphocytes

### 3. PIBF-induced STAT6 activation is mediated by the IL-4 receptor

The activation of STAT6 pathway is initiated uniquely through IL-4R, thus IL-4 and IL-13 are known as the primary activators of STAT6. IL-4R is a heterodimer consisting of the high-affinity  $\alpha$  chain and a trans-activating low-affinity chain, that can be either the common  $\gamma$  or the IL-13R $\alpha$ 1 chain (38). The ligand-induced heterodimerization of IL-4R $\alpha$  and  $\gamma$  chain in lymphoid cells induces the tyrosine phosphorylation of IL-4R $\alpha$  chain and the activation of Jak1 and Jak3 kinases (41). The phosphorylated tyrosine residues of IL-4R $\alpha$  chain serve as anchors for the phosphorylation of STAT6 SH2 domains (42). Based on the assumption that PIBF might be a ligand of IL-4R, Jak- and STAT phosphorylations were tested in PIBF activated lymphocytes that had been pre-treated with the blocking concentrations of anti-IL-4R $\alpha$ , or anti-IL-13R monoclonal antibodies (mAb). The blocking of IL-13R had no effect on PIBF-induced STAT6 phosphorylation, but the use of IL-4R mAb at a suggested concentration for bioactivity neutralization, abolished STAT6 induction (Fig.10).

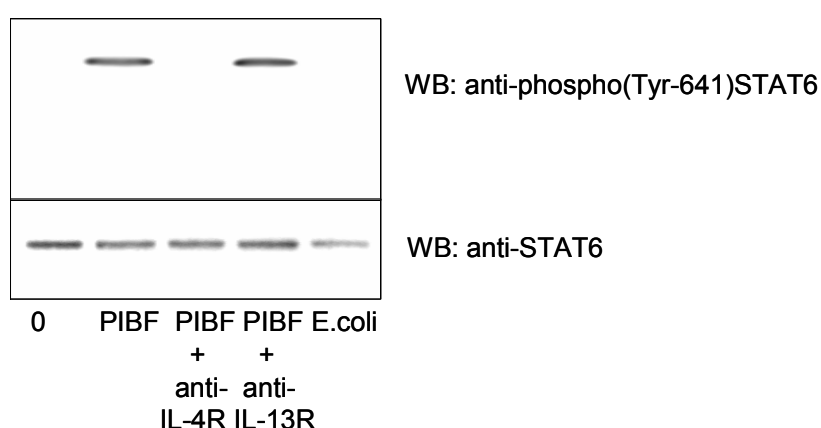


Fig.10 The blocking of IL-4R $\alpha$  inhibits PIBF induced STAT6 phosphorylation

PIBF induced the phosphorylation of IL-4R $\alpha$ -associated Jak1, and this effect was counteracted by anti-IL-4R mAb treatment. The activation of  $\gamma$  chain-associated Jak3 was not detectable after PIBF treatment (Fig.11).

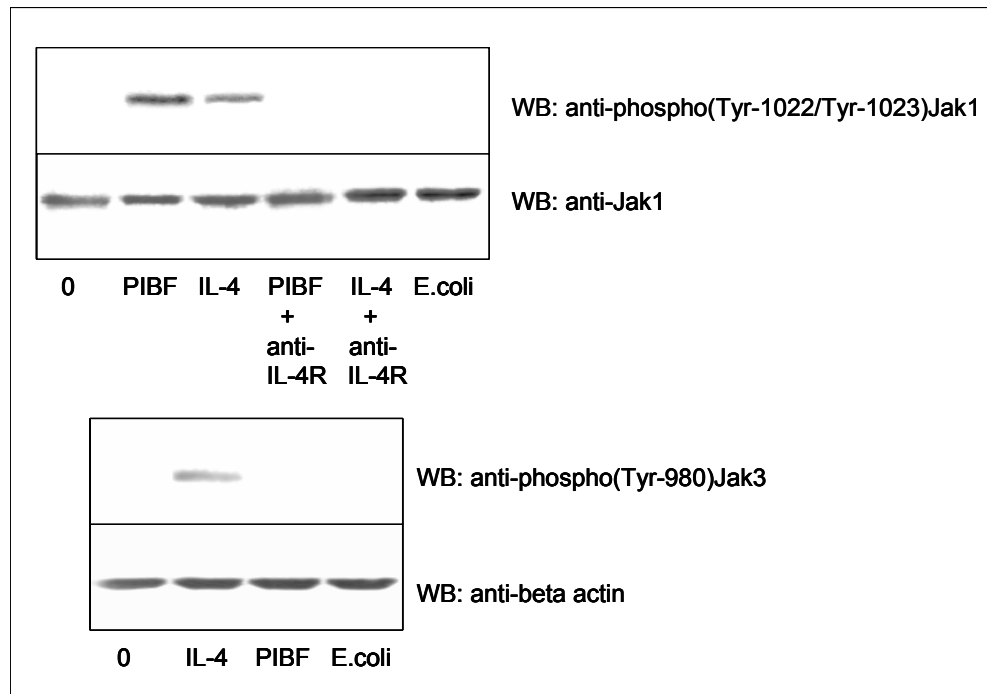


Fig.11 The effect of PIBF on Jak1 and Jak3 induction

Consequently, we attempted to determine, if PIBF is a ligand of IL-4R. We could not demonstrate PIBF binding to IL-4R by ELISA, nor did anti-IL-4R treatment prevent PIBF binding to its own receptor (Fig.12, Fig.13).

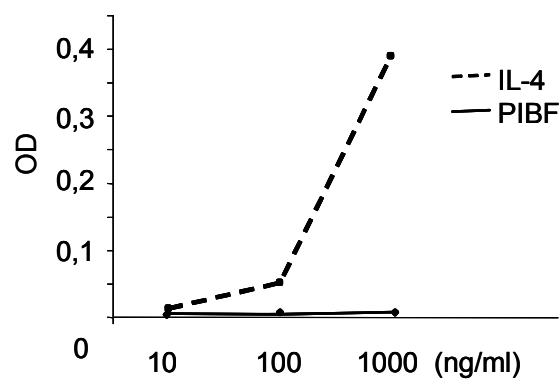


Fig.12 The PIBF binding to IL-4R $\alpha$

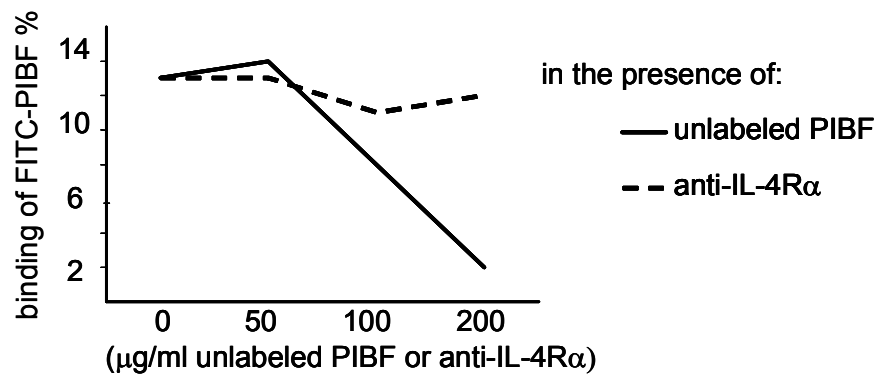


Fig.13 The prevention of PIBF binding to its own receptor by anti-IL-4R $\alpha$  treatment

The possibility that the PIBF antibody binds to a region within PIBF that is required for interaction with the IL4R, thus competing for the same site on PIBF can be ruled out, because in the ELISA we used a polyclonal anti-PIBF IgG which recognizes multiple epitopes on the PIBF molecule. Furthermore, the assay performed in a reverse order (solid phase bound PIBF reacted with IL-4R $\alpha$ , revealed with anti-IL-4R $\alpha$ ) gave similar negative results. Earlier we showed (28) that supernatants from spleen cells activated in the presence of PIBF, produce more IL-4 significantly, than those in the absence of PIBF. The possible scenario that instead of PIBF, PIBF-induced IL-4 would bind to the IL-4R, and phosphorylate STAT6, can also be excluded. Already 1 min incubation with PIBF is sufficient to induce the phosphorylation of STAT6. This is a too short interval for gene induction, plus for the synthesis of IL-4. Another argument against this concept is, that the treatment of the cells with anti-IL-4 antibodies did not inhibit, not even reduce - the effect of PIBF on STAT6 phosphorylation (Paper 4).

Based on the above data, the hypothesis was put forward that upon the ligand binding of PIBF receptor (PIBFR) might form a heterodimer with the alpha chain of the IL-4R, and activate STAT6 pathway. The confocal microscopy analysis of phycoerythrin – (PE)-anti-IL-4R labeled cells (Fig. 14 b) and fluorescein-isothiocyanate – (FITC) PIBF

labeled cells (Fig. 14 a) revealed a co-capping of the two receptors. Fig. 14 c represents the merging of Fig. 14 a. and Fig. 14 b. The co-localization of IL-4R $\alpha$  and PIBFR was performed at 4°C (Fig.14 lower panel) and the co-capping at 37°C (Fig.14 upper panel). For the capping, lymphocytes were incubated with FITC-conjugated PIBF for 30 min at 37 °C, fixed and reacted with monoclonal anti-IL-4R $\alpha$  for 45 min at room temperature, followed by the incubation with PE-labeled rat anti-mouse IgG 2A+B or PE-labeled rat anti-mouse IgG1 for 30 min at room temperature. Below, in the same panel fluorescence- and transmission pictures are merged to show the cellular localization of the receptors.

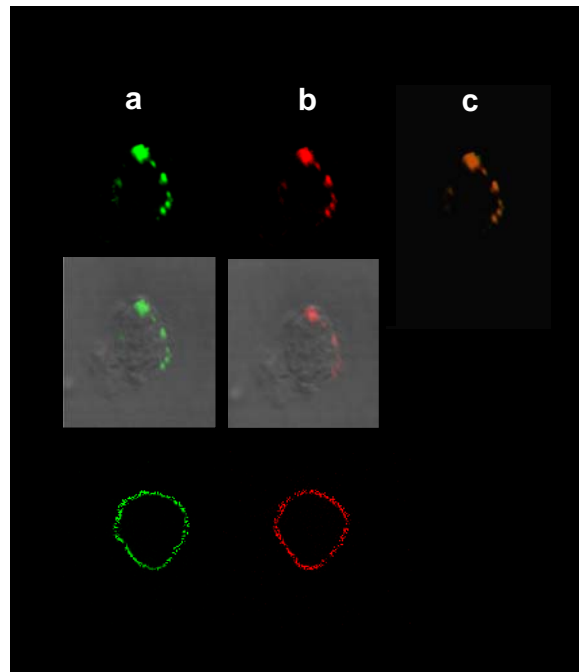


Fig.14 The confocal analysis of IL-4R $\alpha$  and PIBFR co-capping

The above findings raise the question, why does the PIBFR need the IL-4R $\alpha$  for signalling? A plausible explanation would be that the PIBFR itself does not possess an intracellular domain, thus it uses that of IL-4R $\alpha$  .

Several proteins are anchored to membranes via a post-translational lipid modification, the glycosylphosphatidylinositol (GPI) anchor. GPI-anchored proteins have been found in almost all tissues and cells in mammals. In spite of the fact that these molecules have no transmembrane or intracellular domains, ligation of these proteins by antibodies results in signal transduction. Their signalling capacity is due to the association of these molecules with putative transmembrane proteins that can signal via conventional mechanisms (74).

Testing the hypothesis that PIBFR was a GPI anchored protein, we digested the putative anchoring region with phosphatidylinositol-specific phospholipase C (PI-PLC). IL-4 was still able to activate STAT6 in PI-PLC digested cells, but PIBF failed to do so, suggesting that the latter was possibly a GPI anchored protein, indispensable for PIBF to activate STAT6 (Fig.15).

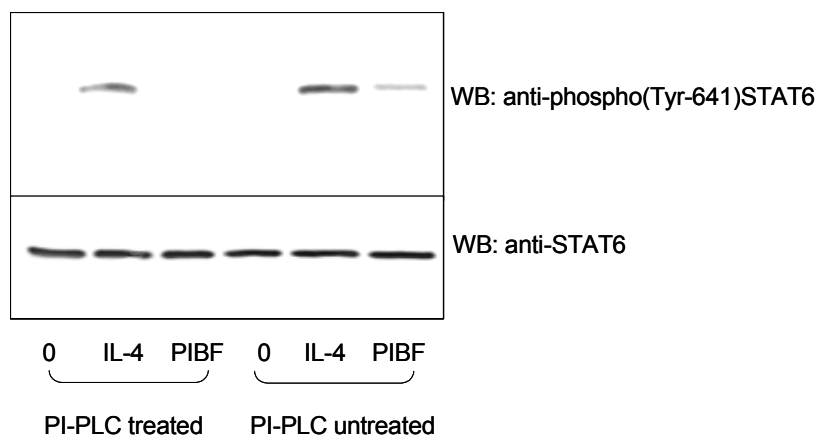


Fig.15 PI-PLC treatment abolishes PIBF induced STAT6 induction

GPI-deficiency has been shown to cause female infertility in mice (75), but to date the GPI-anchored protein needed for maintaining pregnancy has not been identified. Our earlier data show that PIBF depletion ablates murine pregnancy (70), suggesting that

PIBFR might be a considered candidate. The concept that both IL-4R $\alpha$  and PIBFR are required for PIBF signalling is supported by the following:

- 1) Anti-IL-4R $\alpha$  does not prevent binding of PIBF to its receptor, suggesting that PIBFR and IL-4R $\alpha$  are separate entities.
- 2) Digesting the GPI anchor abolishes PIBF driven signalling, thus a GPI-anchored protein is required for PIBF signalling.
- 3) Anti-IL-4R $\alpha$  antibody inhibits PIBF-induced STAT6 phosphorylation in intact cells showing that PIBF can not signal via its own receptor, without the involvement of IL-4R $\alpha$ . Taken together, the GPI-anchored PIBFR, is required, but not sufficient for PIBF signalling.

#### **4. The effects of PIBF on PKC and Ca<sup>++</sup> signalling.**

The development of naive T cells into type 1 or type 2 effector cells is thought to be under the control of cytokines. High levels of PKC activity combined with low Ca<sup>++</sup> signals favour Th2 development, while predominance of Ca<sup>++</sup> signalling with low PKC activity favours Th1 development (54). Signals downstream of PKC and calcineurin directly result in preferential type 1 or type 2 cytokine gene expression (61, 62).

T cells express multiple isotypes of PKC, and though it is accepted that PKCs play a role in T cell activation, little is known about the function of individual PKC isotypes. Therefore we also examined the role of different PKC isotypes in PIBF induced signalling.

Both IL-4 and PIBF treatment induced the phosphorylation of PKC $\alpha/\beta$  II, PKC $\theta$  and PKC $\zeta$ , without affecting intracellular Ca<sup>++</sup> levels (Fig.16).

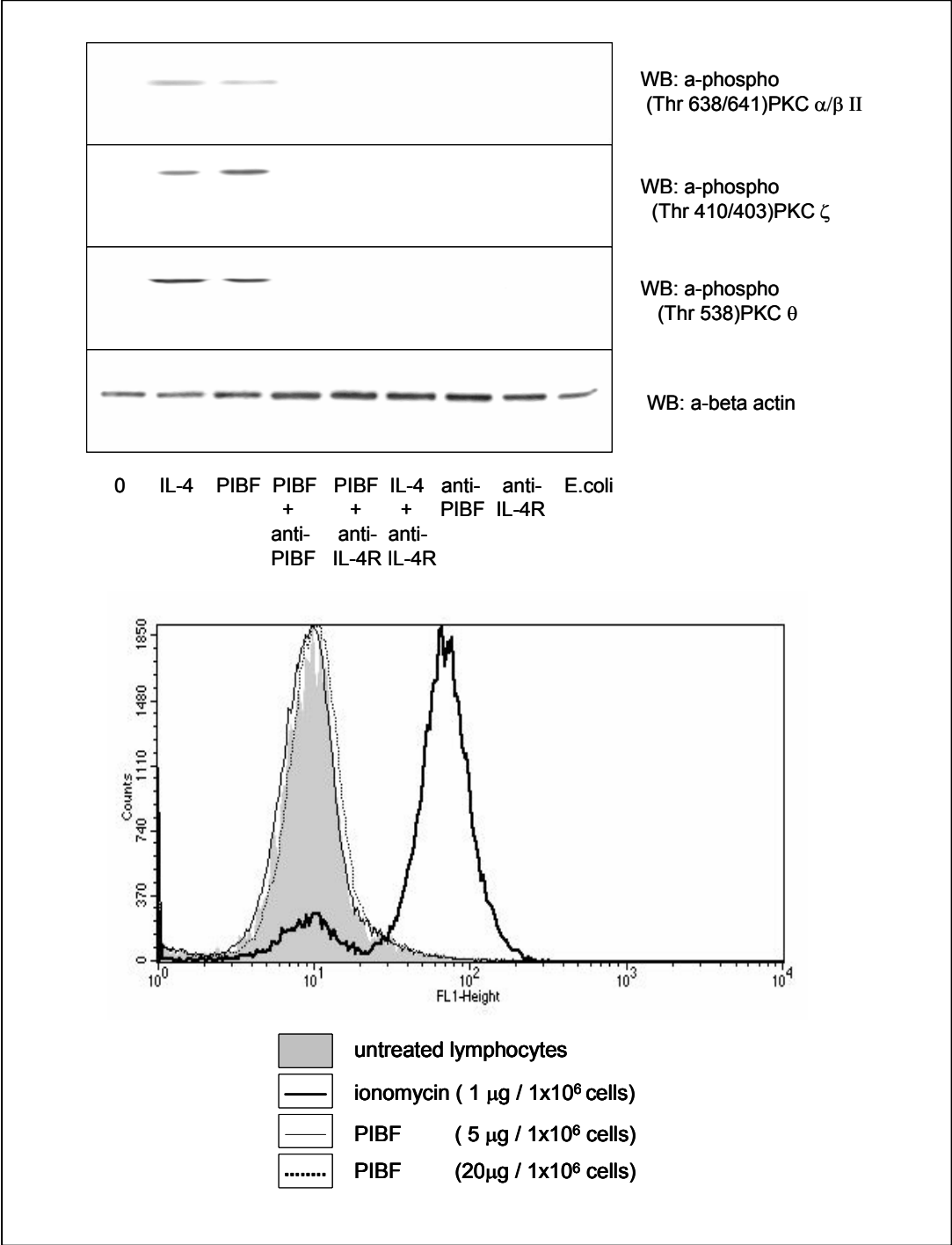


Fig.16 The effects of PIBF on PKC isotypes and intracellular Ca<sup>++</sup> level

The Ca<sup>++</sup> independent, novel PKC isoform PKC $\theta$  and the atypical PKC isoform PKC $\zeta$  are involved in the induction of type 2 development (55, 56), furthermore PKC $\zeta$



activity is required for Jak1 phosphorylation during IL-4 signalling. In this pathway PKC $\zeta$  phosphorylates Jak1, which in turn activates STAT6 (57). Recently published evidence shows that STAT6 is dramatically inhibited in PKC $\zeta$  deficient cells (58).

Since PIBF uses the IL-4R $\alpha$  chain for STAT6 signalling, we tested, whether a functional IL-4R $\alpha$  was required for PKC activation.

The effect of PIBF on the phosphorylation of PKC $\zeta$ , PKC $\theta$ , and PKC $\alpha/\beta$  was abrogated by the pre-treatment of the cells with blocking concentrations of anti-IL-4R $\alpha$  antibody or anti-PIBF IgG, suggesting that both PIBF and functional IL-4R $\alpha$  is needed for PIBF-induced PKC-activation. To test if PKC phosphorylation is needed for the effects of PIBF on Jak1 and STAT6 induction, we blocked PKC activity by isoform specific monoclonal antibodies. The results show that PKC $\zeta$  and PKC $\theta$  phosphorylation, but not that of PKC  $\alpha/\beta$  are required for PIBF-induced Jak1 and STAT6 activation (Fig.17).

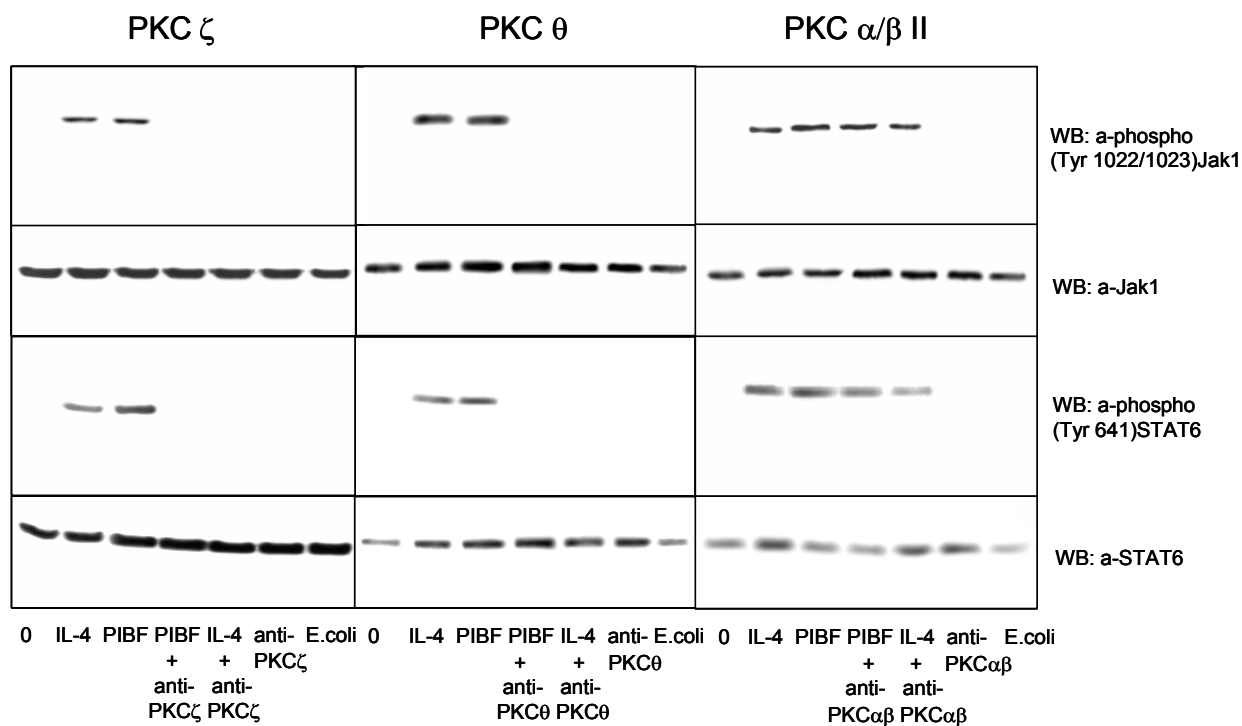


Fig.17 The importance of PKC $\zeta$  and PKC $\theta$  activation in PIBF induced Jak1 and STAT6 phosphorylation

These results suggest that the following interaction of PIBF with IL-4R $\alpha$ , PKC $\zeta$  gets phosphorylated and activates Jak1, which in turn phosphorylates STAT6. Intracellular Ca<sup>++</sup> concentrations at the same time remain unchanged resulting in a Th2 dominant immune response.

## **V. MATERIALS AND METHODS**

### **1. Antibodies and PIBF**

The 48-kDa N-terminal recombinant human PIBF was prepared as described earlier (17).

Polyclonal anti-PIBF antibodies were generated in our laboratory by immunizing rabbits with the 48-kDa N-terminal recombinant human PIBF. The antibody titers were determined by ELISA. IgG was affinity purified on protein A columns.

Monoclonal anti-IL-12 antibody (R&D Systems, Europe Ltd., Abingdon, UK) and HRPO-labeled anti-mouse IgG (Dako, Glostrup, Denmark) were used for immunocytochemistry.

Mouse monoclonal anti-human IL-4 R $\alpha$  as well as goat polyclonal anti-human IL-13 R $\alpha$ 1 antibodies (both from R&D Systems Europe Ltd., Abingdon, UK) were used for treatment of lymphocytes.

Mouse IgG 2A specific Ig (Sigma-Aldrich Inc., Budapest, Hungary), rabbit IgG specific- and goat IgG specific antibodies (both from Dako, Glostrup, Denmark) were used for isotype controls. Rabbit polyclonal phospho-specific (Tyr-641) anti-human STAT6, rabbit polyclonal phospho-specific (Tyr-1022/ Tyr-1023) anti-human Jak1, rabbit polyclonal phospho-specific (Tyr-980) anti-human Jak3, rabbit polyclonal anti-human SOCS1, rabbit polyclonal anti-human SOCS3 antibodies (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit polyclonal phospho-specific (Tyr-693) anti-human STAT4 antibody (Zymed Laboratories Inc., San Francisco, CA, USA) and HRPO-labeled anti-rabbit IgG (Dako, Glostrup, Denmark) were used for Western blotting. Polyclonal rabbit anti-human STAT6, anti-human Jak1, anti-human STAT4 antibodies (all

from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and rabbit anti-human  $\beta$  actin (Sigma-Aldrich Inc., Budapest, Hungary) were used for controlling the loading on Western blots. Rabbit polyclonal phospho-specific (Thr-410/403) anti-human PKC $\zeta$ , phospho-specific (Thr-538) anti human PKC $\theta$ , and phospho-specific (Thr-638/641) anti-human PKC $\alpha/\beta$  II, (all from Cell Signalling Technology, Beverly, MA, USA) were used for blocking and detection of PKC isoforms.

Polyclonal rabbit anti-human STAT6 antibody recommended for gel supershift studies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used for EMSA supershift.

Goat polyclonal anti-human IL-4 (R&D Systems, Europe Ltd., Abingdon, UK), mouse monoclonal anti-human IL-4 R $\alpha$ , HRPO-labeled anti-goat IgG antibody (both from Dako, Glostrup, Denmark), streptavidin-biotinylated HRPO complex (Amersham Biosciences, Little Chalfont, UK) and polyclonal biotin-conjugated anti-PIBF antibodies (prepared in our laboratory) were used for ELISA test.

Mouse monoclonal anti-human IL-4 R $\alpha$ , PE-labeled rat anti-mouse IgG 2A+B (BD Biosciences, San Jose, CA, USA), FITC - conjugated 48-kDa N-terminal recombinant human PIBF (prepared in our laboratory), PE-labeled mouse anti-human CD45RA and PE-labeled rat anti-mouse IgG 1 (both from BD Biosciences, San Jose, CA, USA), were used for immunofluorescence confocal microscopy.

PIBF-FITC and mouse monoclonal anti-human IL-4 R $\alpha$  antibodies were used for determining the receptor binding of PIBF by flow cytometry.

## **2. LPS, Quinacrine and anti-PIBF treatment**

Heparinized venous blood was obtained from 20 healthy pregnant women between the 16<sup>th</sup> and 26<sup>th</sup> week of gestation. Peripheral blood mononuclear cells (PBMC) were separated on a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden) and washed in RPMI 1640 medium (Gibco BRL, Life Technologies, Paisly, Scotland).

LPS (prepared from second phase *Shigella sonnei* in this department) was diluted 10 µg/ml in RPMI 1640 medium. Quinacrine (Sigma-Aldrich Inc., Budapest, Hungary) was dissolved in ethanol and further diluted in RPMI medium from 0.1 to 100 µM concentrations. Stock solutions were always freshly prepared before incubations. PIBF specific polyclonal antibody was prepared in this laboratory as described earlier (20) and used at a concentration of 400 µg/ml.

One million lymphocytes were incubated for 3 hours at 37°C, 5% CO<sub>2</sub> with the following:

- medium (RPMI plus 10% fetal calf serum (FCS))
- medium containing 10 µg/ml LPS
- medium containing LPS and 0.1, 1, 5, 10 and 100 µM quinacrine
- medium containing 400 µg/ml anti-PIBF
- medium containing anti-PIBF and 10 µM quinacrine
- medium containing 10 µM quinacrine or 400 µg/ml pre-immune rabbit polyclonal IgG antibody
- to study the effect of progesterone pre-treatment on LPS treated lymphocytes, cells were incubated with 20 µg/ml progesterone for 24 hours at 37°C, 5% CO<sub>2</sub> prior to LPS incubation.

The toxicity of quinacrine and progesterone treatment were tested on K562 cell line and on lymphocytes using Trypan blue or propidium iodine staining. The viability of K562 cells was not altered by either treatments.

### **3. Immunocytochemistry**

Following the incubation, the cells were washed twice in RPMI 1640 medium and centrifuged on glass microscope slides. The slides were dried at room temperature, the cells were fixed for 5 min in ice cold acetone and washed in tris-buffered saline (TBS). All incubations were carried out at room temperature in a humid chamber. After blocking endogenous peroxidase activity with 1% H<sub>2</sub>O<sub>2</sub> the cells were further incubated in TBS containing 1% bovine serum albumin (BSA) (Sigma-Aldrich Inc., Budapest, Hungary) for blocking non-specific protein binding.

The primary anti-IL-12 monoclonal antibody (from R&D Systems, Europe Ltd. Abingdon, UK) was diluted 1:50 in TBS supplemented with 0.5% BSA and the cells were incubated for 1 hour. The secondary HRPO-labeled anti-mouse IgG (from Dako, Glostrup, Denmark) were applied at a dilution of 1:100 for 30 min. The reaction was developed by diaminobenzidine (Sigma-Aldrich Inc., Budapest, Hungary) followed by silver intensification. The nuclei were counter-stained with hematoxylin and the slides were mounted with gelatin-glycerol. The slides were read blind and the percentage of positive cells was determined after counting 500 lymphocytes in the microscope at high power magnification.

Statistics: The two tailed Student's t-test was used for statistical evaluation of the data. Differences were considered significant if P-value was equal or less than 0.05.

#### **4. Treatment of lymphocytes for examination of signalling pathways**

Lymphocytes of healthy non-pregnant volunteers were isolated from heparinized venous blood on Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden), and washed in RPMI 1640 medium (Gibco BRL, Life Technologies, Paisly, Scotland). The cells were treated with 1µg/ml of Phytohemagglutinin (PHA) (Sigma-Aldrich Inc., Budapest, Hungary) for 24 hours and then incubated at 37°C in 5% CO<sub>2</sub> with the following:

- RPMI medium 1640 (control),
- different concentrations (10ng/ml, 20ng/ml, 200ng/ml, 0,5µg/ml, 1µg/ml, 10µg/ml for  $5 \times 10^7$  cells) of the 48-kDa N-terminal recombinant human PIBF
- PIBF, progesterone (Sigma-Aldrich Inc., Budapest, Hungary) or recombinant human IL-4 (R&D Systems Europe Ltd., Abingdon, UK) (for 1, 10, 20, 30 minutes and 24 hours),
- recombinant human IL-4 together with recombinant human IL-12 (R&D Systems Europe Ltd., Abingdon, UK), or with recombinant human IL-12 in the presence of monoclonal anti-human IL-4Rα antibody or with monoclonal anti-human IL-4Rα antibody.
- 48-kDa N-terminal recombinant human PIBF and polyclonal anti-PIBF antibody, or monoclonal anti-human IL-4Rα antibody, or recombinant human IL-12 together with monoclonal anti-human IL-4Rα antibody, or recombinant human IL-12 together with monoclonal anti-human IL-13Rα1 antibody
- recombinant human IL-12 and 48-kDa N-terminal recombinant human PIBF, or recombinant human IL-4
- recombinant human PIBF together with phospho-specific anti-human PKCζ, or with phospho-specific PKCθ, or with phospho-specific anti-human PKCα/β II.

- recombinant human IL-4 together with phospho-specific anti-human PKC $\zeta$ , or with phospho-specific PKC $\theta$ , or with phospho-specific anti-human PKC $\alpha/\beta$  II.
- lysate of E. coli that had undergone the same purification procedure as the recombinant human PIBF in a concentration of 200ng/ml for  $5 \times 10^7$  cells.

The phospho-specific PKC antibodies were diluted 1:100 for the treatments in blocking concentrations. To determine the specificity of the reactions, isotype controls were used with each treatment. The cytokines were used at a concentration of 200ng/ml for  $5 \times 10^7$  cells, whereas anti-human IL-4R $\alpha$  and anti-human IL-13R $\alpha$ 1 antibodies were applied in a blocking concentration (50 $\mu$ g/ml for  $5 \times 10^7$  cells). All treatments were performed for 20 min. The pre-treatment of lymphocytes with the PKC inhibitor, Staurosporine (Sigma Aldrich Inc, Budapest, Hungary) was performed at a concentration of 400ng/ml for  $5 \times 10^7$  cells at 37°C in 5% CO<sub>2</sub> for an hour.

## **5. Western blotting**

Following the treatment, lymphocytes were washed in PBS and then in PBS containing 1mM sodium-vanadate and 5mM NaF by centrifugation. Samples were resuspended and centrifuged at 13000 rpm for 40 sec. Pellets were resuspended in 3 volumes of buffer containing 20 mM HEPES (pH 7.9), 20 mM NaF, 1mM sodium-vanadate, 1 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1mM EDTA, 1mM EGTA, 5mM dithiothreitol (DTT), 0.5mM PMSF, aprotinin (0.01 $\mu$ g/ml), leupeptin (0.025 $\mu$ g/ml), 400mM NaCl, 20% glycerol. Samples were frozen and thawed three times, and kept on ice for a further 20 min. After



centrifugation at 15000 rpm at 4°C for 30 min., the supernatants were collected, and the protein content of the extracts was determined.

For the detection of different transcription factors in the extracts of lymphocytes, 25µg of total protein was separated by 7.5% or 12% SDS-PAGE, and transferred to a Hybond ECL membrane (Amersham Biosciences, Little Chalfont, UK) overnight at 54 mA. The blots were blocked with 3% non-fat dry milk in TBS-Tween solution for an hour, then incubated with 1:20000 diluted rabbit polyclonal phospho-specific (Tyr-641) anti-human STAT6 or with 3 µg/ml rabbit polyclonal phospho-specific (Tyr-693) anti-human STAT4 or with 1:2000 diluted rabbit polyclonal phospho-specific (Tyr-1022/ Tyr 1023) anti-human Jak1 or with 1:200 diluted rabbit polyclonal phospho-specific (Tyr-980) anti-human Jak3 or with 1:200 diluted rabbit polyclonal anti-human SOCS1 or with 1:200 diluted rabbit polyclonal anti-human SOCS3 antibodies in TBS-Tween solution containing 3% non-fat dry milk for an hour.

The blots for detecting PKC factors were blocked with 3% non-fat dry milk in TBS-Tween solution for an hour, then incubated with 1:1000 diluted rabbit polyclonal phospho-specific anti-human PKCζ or phospho-specific anti-human PKCθ or phospho-specific anti-human PKCα/β II for 4 hours in TBS-Tween solution containing 3% non-fat dry milk.

For controlling the loading, 1:1000 diluted rabbit polyclonal anti-human STAT6 or 1:1000 diluted rabbit polyclonal anti-human STAT4 or 1:1000 diluted rabbit polyclonal anti-human Jak1 antibodies or 1:1000 diluted rabbit anti-human β actin antibodies were used. After six washing cycles (10 min. each) in TBS-Tween (pH 7.4), the blots were incubated at room temperature for 45 min with 1:2000 diluted HRPO-labeled anti-rabbit IgG, and then washed six times (10 min. each) in TBS-Tween. Antibody binding was

detected using an ECL kit (Perkin Elmer Life Sciences, Boston, MA, USA) according to the manufacturer's instructions.

## **6. EMSA Supershift**

Nuclear extracts were prepared as described by Xu and Cooper (76) and all subsequent steps were performed at 4 °C. Following the treatment, lymphocytes were washed twice in ice cold phosphate buffered saline (PBS) and resuspended in 10 volumes of buffer containing 10mM HEPES (pH 7.9), 1.5mM MgCl<sub>2</sub>, 10mM KCl, 0.5 mM DTT, protease inhibitors (Complete, Mini EDTA-free tablets, Boehringer Mannheim, Mannheim, Germany), phosphatase inhibitors (Phosphatase Inhibitor Cocktail, Sigma-Aldrich Inc., Budapest, Hungary) and placed on ice for 10 min. After vigorous vortexing, nuclei were collected by centrifugation in microcentrifuge at 10000 rpm for 10 seconds, resuspended in 2 volumes of buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitors, phosphatase inhibitors and placed on ice for 20 min. After centrifugation in a microcentrifuge at 10000 rpm for 10 seconds, the supernatants were collected, the protein content of the extracts was determined, aliquoted and stored at -80 °C. For shift assay analysis STAT6 binding probes (5' TCGACTTCCCAAGAACAGCA 3') and their reverse complementary pairs were incubated at 80 °C for 10 min. in annealing buffer containing 100 mM NaCl, 10 mM TRIS (pH 7.5), 1mM EDTA and allowed to cool down slowly to room temperature. Double-stranded oligonucleotides were end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase (Promega Corp. Madison, WI USA) according to the manufacturer's protocol. Binding reactions were performed at room temperature for 30 min. in 20μl volume, containing 300ng polydIdC, 100mM NaCl, 0.25mM EDTA,

1mM DTT, 20mM HEPES (pH 7.9) and 20 fmol <sup>32</sup>P-labeled oligonucleotide probe. For supershift assays the reaction mixtures were pre-incubated with 1µl rabbit anti-human STAT6 IgG (2µg/µl) for 1 h at 4 °C before adding the <sup>32</sup>P-labeled oligonucleotide. Samples were separated on 3.5% acrylamide gel with 0.5x Tris-Borate-EDTA buffer. The gel was dried and exposed to X-ray film.

## **7. Phosphatidylinositol-Specific Phospholipase C treatment**

After isolation, washing and PHA treatment of lymphocytes, as described above, (5x10<sup>6</sup> cells/ samples) were incubated with 2.5 Units of PI-PLC (Sigma-Aldrich Inc., Budapest, Hungary) in 1 ml of PBS for 30 minutes at 37°C in 5% CO<sub>2</sub> and washed twice with PBS by centrifugation.

## **8. ELISA test for detecting binding of PIBF to IL-4R**

During an overnight incubation at 4 °C, 96 well-microtiter plates were coated with 100 µl/well of 1 µg/ml and 0.5 µg/ml of soluble recombinant human IL-4Rα (R&D Systems Europe Ltd., Abingdon, UK) in 50 mM carbonate buffer (pH 9.6). All further incubations were performed at 37°C. Plates were washed three times and free binding sites were blocked with PBS containing 0.05% Tween, 0.5% gelatin and 0.1% BSA for an hour. The logarithmic dilutions (0.01-1 µg/ml) of 48-kDa N-terminal recombinant human PIBF or recombinant human IL-4 in PBS Tween (pH 7.4) were incubated on the plates for 60 min. The plates were washed 3 times, and incubated with 1:1000 diluted biotin-conjugated anti-PIBF antibody or 1:1000 diluted goat anti-human IL-4 antibodies for an hour. Following 3 further washing cycles, biotin-conjugated anti-PIBF IgG was reacted

with 1:1000 diluted streptavidin-biotin-HRPO (Amersham Biosciences, Little Chalfont, UK) for 30 min., whereas anti-IL-4 antibody was reacted with 1:2000 diluted HRPO-labeled anti-goat IgG for 60 min. The latter was then incubated with 1:1000 diluted streptavidin-biotin HRPO for 30 min. The reaction was developed with O-phenylenediamine (ODP) (FLUKA, Sigma-Aldrich Inc., Budapest, Hungary) and the reaction was stopped by adding 50 µl of 4M H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 490 nm.

## **9. Determination of receptorbinding of PIBF by flow cytometry**

One million peripheral lymphocytes from healthy volunteers were incubated with 2 µg/ml FITC-conjugated PIBF in the presence of increasing concentrations (0-20 µg/100 µl) of unlabelled PIBF or monoclonal anti-human IL-4R $\alpha$  antibody for 30 min. at 4°C. After the incubation, the cells were washed in PBS then fixed in 0.5 ml 4% PBS-formalin and analyzed by flow cytometry, using a FACSCalibur flow cytometer (BD Immunocytometry Systems, BD Biosciences, San Jose, CA, USA) equipped with a 488 nm excitation laser with the CellQuest software program (BD Biosciences, San Jose, CA, USA).

## **10. Confocal microscopy**

One million peripheral lymphocytes from healthy volunteers were incubated with 5 µg of FITC-conjugated PIBF for 20 min at 37 °C. The cells (1x10<sup>5</sup>) were plated on poly-L-lysine coated slides and incubated at 37 °C for further 10 min., then washed twice in PBS containing 1% FCS and fixed with freshly prepared 3 % paraformaldehyde in PBS for 10 min. at room temperature. After washing, the plates were incubated with 0.5 µg of

monoclonal anti-human IL-4R $\alpha$  or 2  $\mu$ l of PE-labeled mouse anti-human CD45RA antibody for 45 min. at room temperature. Cells were washed twice and incubated with 2  $\mu$ l PE-labelled rat anti-mouse IgG 2A+B or PE-labeled rat anti-mouse IgG1 for 30 min. at room temperature. Cells were washed twice and the slides were mounted with DABCO (Sigma-Aldich Inc., Budapest, Hungary). To control the specificity of the capping formation, all steps were also performed at 4°C. The slides were analyzed with a Biorad confocal microscope with 100x objective, using laser excitation at 473 nm and filters 580 $\pm$ 16 nm for PE and 522 $\pm$ 17.5 nm for FITC. Images were analysed using the Adobe Photoshop 7.0 program.

## **11. RNA interference (RNAi) and Cytometric Bead Arrays**

Oligonucleotides were hand-designed to interfere exclusively with STAT6 mRNA (Ambion Inc., Huntington, UK). As negative control, the very same nucleotides were scrambled to form a non-genomic combination (controlled by Pubmed Blast search). Oligonucleotide sequences used for STAT6 RNAi were the following:

- Target Sequence for STAT6 AAG CAG GAA GAA CTC AAG TTT
- Target Sequence Scrambled AAA CGA GAG TGT TAT AAC TGT

Cells were washed twice with Optimem media (Invitrogen, Karlsruhe, Germany). Oligonucleotides were dissolved in RNase-free water and annealed according to the guidelines provided by the manufacturer, and incubated for 20 min. at room temperature with Oligofectamine (Invitrogene Karlsruhe, Germany). This mixture was added drop-wise to the cells, until a concentration of 66 nM was reached. After 4 hours incubation at 37°C, DMEM containing 30% FCS was added to cultures. Western blots for STAT6 expression were performed 24 hours subsequent to siRNA transfection.

Lymphocytes culture: Lymphocytes (intact, control treated with scrambled oligos and those after RNAi) were adjusted to  $10^6$  cells/ml in RPMI1640 medium containing 10% FCS, and cultured for 48 hours at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere with 0.2 or 10 µg/ml of the 48 kDa N-terminal recombinant human PIBF or without PIBF. Supernatants were harvested for cytokine determination. Lymphocytes were lysed for Western blot analysis.

Cytometric Bead Arrays (CBA; Becton Dickinson, San Jose, CA, USA or Bender MedSystems, Vienna, Austria) were used to analyze the concentrations of IL-10, TNFα and IFNγ in the supernatants of lymphocyte cultures. Supernatants were incubated with labelled capture beads and detection reagent for 3 hours in the dark at room temperature, and analyzed with flow cytometer (FACScalibur, Becton Dickinson, San Jose, CA, USA for BD arrays or alternatively Galaxy, Dako, Denmark) by using the respective CBA Analysis Software (BD, San Jose, CA, USA) and Bender MedSystem (Bender MedSystems, Vienna, Austria) software.

Statistical analysis: The two-tailed Student's t-test was used for statistical evaluation of the data. Differences were considered significantly if the p value was equal to or lower than 0.05.

## **12. Flow cytometric determination of intracellular Ca<sup>++</sup>**

In peripheral lymphocytes from healthy non-pregnant volunteers intracellular free Ca<sup>++</sup> was measured using Fluo-3 AM according to the protocol described by Minta et al (77, 78). Briefly,  $10^6$  cells were incubated for 30 min at room temperature in 100µl tissue culture medium containing 10µM Fluo-3 AM. The cell suspensions were diluted with 10

ml RPMI containing 10% FCS and incubated for further 30 min. Samples were washed twice in RPMI containing 10% FCS. Activation was carried out with ionomycin ( $1\mu\text{g}/10^6$  cells) as a non-specific activator (79, 80) or 48-kDa N-terminal recombinant human PIBF ( $5\mu\text{g}/10^6$  and  $20\mu\text{g}/10^6$  cells). 7-Aminoactinomycin D (7-AAD) labelling was used to distinguish dead cells by their higher fluorescence at 647 nm (FL3 channel). The viable cells were gated and all further calculations were performed excluding the 7-AAD positive cells. Increase of fluorescence was measured in a Becton Dickinson FacsCalibur flow cytometer using the CellQuest program. The mean fluorescence intensity of Fluo-3 AM dye (proportional to the cytosolic calcium level) was determined at 526 nm (FL1 channel). After measuring basal fluorescence at 526 nm, the activating agent was added and the measurement continued for further 100 to 400 seconds, enabling us to follow the alterations of intracellular free  $\text{Ca}^{++}$  level (FL1 fluorescence intensity) in function of time. Gates were created along the time axis of the activation dot plots at definite time points and the mean fluorescence intensity at 526 nm was statistically analyzed from each gate.

## **VI.   THESES**

The present data show that neutralization of endogenously produced PIBF by anti-PIBF antibody results in a more than threefold increase in the percentage of IL-12 positive mononuclear cells, which is corrected by simultaneous Q treatment, suggesting, that PIBF inhibits IL-12 production via an action on AA metabolism.

The possible mechanism might be suggested that the progesterone binding of lymphocytes is followed by the release of PIBF that inhibits AA release. The subsequent block of PG synthesis reduces IL-12 production and results in lowered NK activity, which favours a normal pregnancy outcome.

According to the findings in this study we suggest the existence of a novel type of IL-4R, where, upon ligation the PIBFR combines with the alpha chain of the IL-4R, and induces the phosphorylation of Jak1, which in turn phosphorylates STAT6. Phosphorylated STAT6 dimers move to the nucleus, where they activate the expression of different genes, probably the SOCS3, which via binding to the IL-12R inhibits the phosphorylation of STAT4.

Testing the hypothesis that the PIBFR was a GPI anchored protein, we digested the anchoring region with PI-PLC. In PI-PLC digested cells, IL-4 was still able to activate STAT6, but PIBF failed to do so, suggesting that the latter was a GPI anchored protein, indispensable for PIBF to activate STAT6. GPI-deficiency has been shown to cause female infertility in mice, but to date the GPI-anchored protein needed for maintaining pregnancy has not been identified. Our earlier data show that PIBF depletion ablates murine pregnancy, suggesting that the PIBFR might be a considered candidate.



The above findings would explain the previously described cytokine effects of PIBF (28), as well as its pregnancy protective (70) and possible tumor promoting effects (16).

In this study we also show that PIBF, which induces a Th2 dominant cytokine pattern, activates the PKC signal transduction pathway. T cells express multiple isoforms of PKC, and though it is accepted that PKCs play a role in T cell activation, little is known about the function of individual PKC isoforms. Our results show that the  $Ca^{++}$  independent, novel protein kinase C isoform PKC $\theta$  and the atypical protein kinase C isoform  $\zeta$  are involved in the Th2 biased immune response induced by PIBF.

Earlier data from our laboratory show that PIBF signals via the IL-4R and this results in phosphorylation of Jak1 and STAT6. PKC $\zeta$  activity is required for Jak1 function, and in PKC $\zeta$  deficient cells phosphorylation of STAT6 is dramatically inhibited. We recently demonstrated increased PKC $\zeta$  phosphorylation following PIBF treatment, and showed that PKC $\zeta$  and PKC $\theta$  phosphorylation but not that of PKC  $\alpha/\beta$  are required for PIBF-induced Jak1 and STAT6 activation.

The first event of PIBF signalling is possibly the phosphorylation of PKC $\zeta$ , which depends on ligation of the IL-4R. Indeed, blocking of the IL-4R $\alpha$  counteracted the PKC activating effect of PIBF. Based on these data we propose the following mechanism; following interaction of PIBF with IL-4R $\alpha$ , PKC $\zeta$  gets phosphorylated and activates Jak1, which in turn phosphorylates STAT6.

Th1 activation is characterized by increased intracellular  $Ca^{++}$  concentrations and low PKC signal, while little or no elevation of intracellular free  $Ca^{++}$  concentration occurs in activation of Th2 cells via TCR complex compared to Th1 cells. In line with the finding, that progesterone does not affect lymphocyte  $Ca^{++}$  channels, PIBF induced PKC phosphorylation, without any effect on intracellular  $Ca^{++}$  levels.

High PKC activity and low intracellular  $\text{Ca}^{++}$  levels favour the development of Th2 cytokine sensitive cells. These findings with our earlier data - showing STAT6 activation by PIBF - might account for the Th2 biased immune response induced by PIBF.

## **VII. PAPERS**

## VIII. REFERENCES

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## **IX. LIST OF PUBLICATIONS**

### **Paper 1**

Progesterone regulates IL-12 expression in pregnancy lymphocytes by inhibiting Phospholipase A2

G. Par, J. Geli, N. Kozma, P. Varga, J. Szekeres-Bartho

American Journal of Reproductive Immunology, 2003;49:p:1-5.

### **Paper 2**

Progesterone-dependent immunomodulation

J. Szekeres-Bartho, B. Polgar, N. Kozma, E. Miko, G. Par, L. Szereday, A. Barakonyi, T. Palkovics, O. Papp, P. Varga

Chemical Immunology and Allergy, 2005;89:118-125

### **Paper 3**

The Progesterone-Induced Blocking Factor modulates the balance of PKC and intracellular Ca<sup>++</sup>

N. Kozma, M. Halasz, T. Palkovics, J. Szekeres-Bartho

American Journal of Reproductive Immunology (in press)

### **Paper 4**

Progesterone-Induced Blocking Factor activates STAT6 via binding to a novel IL-4 receptor

N. Kozma, M. Halasz, B. Polgar, T. G. Poehlmann, U. R. Markert, T. Palkovics, M. Keszei, G. Par, K. Kiss, J. Szeberényi, L. Grama, J. Szekeres-Bartho

The Journal of Immunology (in press)









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# Progesterone Regulates IL12 Expression in Pregnancy Lymphocytes by Inhibiting Phospholipase A2

Par G, Geli J, Kozma N, Varga P, Szekeres-Bartho J. Progesterone regulates IL12 expression in pregnancy lymphocytes by inhibiting phospholipase A2. AJRI 2003; 49:1-5 © Blackwell Munksgaard, 2003

**PROBLEM:** Progesterone-induced blocking factor (PIBF) is one of the pathways that mediate the immunological effects of progesterone. PIBF inhibits natural killer (NK) cytotoxic activity. Recently we showed that neutralization of PIBF results in an increased interleukin (IL)-12 expression, which is corrected by cyclooxygenase inhibitors. As exogenous arachidonic acid (AA) voids the NK blocking effect of PIBF, it is likely that PIBF acts before the level of the cyclooxygenase enzyme. Therefore in this study we investigated the effect of PIBF neutralizing antibody and simultaneous phospholipase A2 inhibitor quinacrine (Q) treatment on IL-12 production.

**METHODS:** Pregnancy lymphocytes were treated with anti-PIBF antibody or lipopolysaccharide (LPS) as a positive control, in the presence or absence of Q. IL-12 expression by PBMC was detected by immunocytochemistry.

**RESULTS:** Neutralization of PIBF as well as LPS treatment resulted in an increased IL-12 expression, which was corrected by simultaneous Q treatment. Pre-treatment of lymphocytes with progesterone prevented the stimulating effect of LPS on IL-12 production.

**CONCLUSION:** Progesterone binding of the lymphocytes is followed by the release of PIBF that inhibits AA release. The subsequent block of prostaglandin synthesis reduces IL-12 production and results in a lowered cytotoxic NK activity, which may contribute to a normal pregnancy outcome.

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**Key words:** IL-12, LPS, phospholipase A2, progesterone, quinacrine

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## INTRODUCTION

In the presence of progesterone, pregnancy lymphocytes synthesize a 34-kDa immunomodulatory protein, named the progesterone-induced blocking factor (PIBF).<sup>1</sup> PIBF inhibits natural killer (NK) activity *in vitro*<sup>2</sup> and exerts an antiabortive effect *in vivo*. Neutralization of endogenous PIBF in pregnant mice results in abortion, via increased NK activity, while NK mediated resorptions are counteracted by a simultaneous PIBF treatment.<sup>3</sup> The capacity of the lymphocytes to produce PIBF is related to the outcome of human pregnancy.<sup>4</sup>

All decidual CD56+ cells produce PIBF<sup>5</sup> and – in spite of their high perforin content – show a low rate of cytotoxicity.<sup>6</sup> PIBF maintains low NK activity via

multiple mechanisms. Recent data from our laboratory suggest that PIBF inhibits degranulation and perforin liberation from NK cells and peripheral lymphocytes<sup>5</sup> thus it cannot be excluded that locally produced PIBF contributes to low decidual NK activity. PIBF also affects cytokine secretion by *in vitro* activated lymphocytes.<sup>7</sup> By increasing interleukin-10 and decreasing IL-12 production, PIBF inhibits NK cell cytotoxicity.<sup>7</sup>

Pro-inflammatory cytokines modulate prostaglandin (PG) output through effects on PG synthesis and metabolism. These effects may be opposed by anti-inflammatory cytokines. IL-1 $\beta$ , IL-6 and TNF- $\alpha$  will activate cells to increase PG synthesis by induction of cytosolic phospholipase A2 (PLA2) and COX2 messenger RNA (mRNA) as well as protein expression in amnion-, decidual- and myometrial cells.<sup>8</sup> Increased

amniotic cytokine and PG production is associated with a positive amniotic culture and also with pre-term delivery.<sup>9</sup> The stimulatory effect of pro-inflammatory cytokines is down regulated by progesterone.<sup>10</sup>

Earlier observations revealed a relationship between PGF2 $\alpha$  levels, progesterone binding capacity and cytotoxic activity of the lymphocytes.<sup>11</sup> In a recent study we observed a significantly increased IL-12 production in anti-PIBF treated pregnancy lymphocytes, which was corrected by cyclooxygenase inhibitor (indomethacine) treatment.<sup>12</sup> It is likely that PIBF acts before the level of the cyclooxygenase and lipooxygenase enzymes, as the blocking effect on cytotoxic activity is voided in the presence of exogenous arachidonic acid (AA).<sup>1</sup> Therefore, in the present study we investigated the effect of PIBF neutralizing antibody and simultaneous PLA2 inhibitor quinacrine (Q) treatment on IL-12 production.

## MATERIALS AND METHODS

### *Treatment of Peripheral Blood Mononuclear Cells by LPS, Quinacrine and Anti-PIBF Antibody*

Heparinized venous blood was obtained from 20 healthy pregnant women between the 16th and 26th week of gestation. Peripheral blood mononuclear cells (PBMC) were separated on a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden) and washed in RPMI1640 medium (GibcoBRL, Life Technologies, Paisley, Scotland). Lipopolysaccharide (prepared from second phase *Shigella sonnei* in this Department) was diluted 10  $\mu\text{g}/\text{mL}$  in RPMI 1640 medium. Quinacrine (Sigma Chemical Co., St Louis, MO, USA) was dissolved in ethanol and further diluted in RPMI medium from 0.1 to 100  $\mu\text{M}$  concentrations. Stock solutions were always freshly prepared before the incubations. PIBF specific polyclonal antibody was prepared in this laboratory as described earlier<sup>4</sup> and used at a concentration of 400  $\mu\text{g}/\text{mL}$ . One million lymphocytes were incubated for 3 hr at 37°C, 5% CO<sub>2</sub> with the following: (a) medium (RPMI medium plus 10% fetal calf serum); (b) medium containing 10  $\mu\text{g}/\text{mL}$  LPS; (c–g) medium containing LPS and 0.1, 1, 5, 10 and 100  $\mu\text{M}$  quinacrine; (h) medium containing 400  $\mu\text{g}/\text{mL}$  anti-PIBF; (i) medium containing anti-PIBF and 10  $\mu\text{M}$  quinacrine; (j–k) medium containing 10  $\mu\text{M}$  quinacrine or 400  $\mu\text{g}/\text{mL}$  pre-immune rabbit polyclonal IgG antibody were used as controls; (l) to study the effect of progesterone pre-treatment on LPS treated lymphocytes, cells were incubated with 20  $\mu\text{g}/\text{mL}$  progesterone for 24 hr at 37°C, 5% CO<sub>2</sub> prior to LPS incubation.

The toxicity of the quinacrine and progesterone treatment were tested on K562 cell line and on

lymphocytes using Trypan blue or propidium iodine staining. The viability of K562 cells was not altered by either treatments.

### *Immunocytochemistry*

At the end of the incubation the cells were washed twice in RPMI 1640 medium and centrifuged on glass microscope slides. The slides were dried at room temperature, the cells were fixed for 5 min in ice cold acetone and washed in tris-buffered saline (TBS). All incubations were carried out at room temperature in a humid chamber. After blocking endogenous peroxidase activity with 1% H<sub>2</sub>O<sub>2</sub> the cells were further incubated in TBS containing 1% bovine serum albumin (BSA, Sigma Chemical Co.) for blocking non-specific protein binding.

The primary antibody was anti-IL-12 monoclonal antibody (purchased from R&D Systems, Abingdon, Oxon, UK). The antibody was diluted 1:50 in TBS supplemented with 0.5% BSA and the cells were incubated for 1 hr. Secondary antibodies (HRPO labeled antimouse IgG purchased from Dako A/S, Denmark) were applied at dilution of 1:100, respectively, for 30 min. The reaction was developed by diaminobenzidine (Sigma Chemical Co.) followed by silver intensification. The nuclei were counter-stained with hematoxylin and the slides were mounted with gelatin-glycerol. The slides were read blind and the percentage of positive cells was determined after counting 500 lymphocytes in the microscope at high power magnification.

### *Statistics*

The two tailed Student's *t*-test was used for statistical evaluation of the data. Differences were considered significant if *P*-value was equal or less than 0.05.

## RESULTS

### *The Effect of LPS and Quinacrine on IL-12 Production*

LPS selectively stimulates PG synthesis and enhances cytotoxicity.<sup>16</sup> In our hands, LPS treatment significantly increased the percentage of IL-12 positive cells, and the specific cyclooxygenase inhibitor, indomethacine, corrected this effect.<sup>17</sup> In the present experiment we investigated the effect of a specific PLA2 inhibitor; quinacrine on LPS-induced IL-12 production. One million lymphocytes were incubated with LPS in the presence or absence of 10  $\mu\text{M}$  of quinacrine. Another sample was pre-incubated for 24 hr with 20  $\mu\text{g}/\text{mL}$  of progesterone prior to addition of LPS. Quinacrine decreased LPS-induced IL-12 production in a concentration dependent fashion (Fig. 1). Quinacrine at a

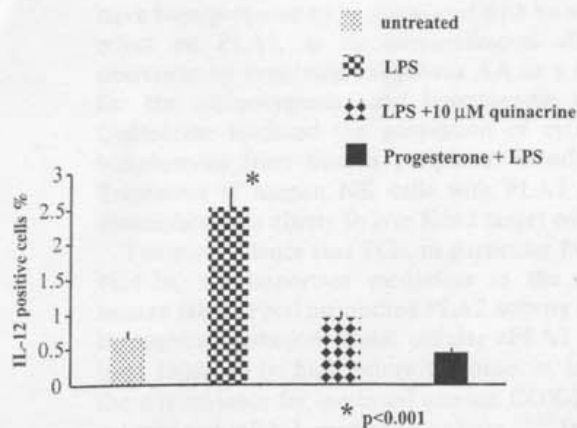


Fig. 1. The effect of quinacrine or progesterone on IL-12 expression of LPS-treated human peripheral mononuclear cells. The bars represent the mean  $\pm$  S.E.M. of 20 determinations. \*Significantly different from the control (untreated) at  $P < 0.001$ .

concentration of 10  $\mu$ M completely abrogated the effect of LPS on IL-12 production. Twenty-four hours pretreatment of lymphocytes with 20  $\mu$ g/mL of progesterone prevented the stimulating effect of LPS on IL-12 production (Fig. 2).

*The Effect of Anti-PIBF Antibody and Quinacrine Treatment on IL-12 Production by Pregnancy PBMC*  
PIBF induces a T helper type 2 (Th2) cytokine response.<sup>7</sup> In the present experiment we investigated the effect of PIBF neutralizing antibody and simultaneous quinacrine treatment on IL-12 expression by PBMC. Lymphocytes were incubated with a polyclonal anti PIBF antibody in the presence or absence of quinacrine. In controls irrelevant antibody was used

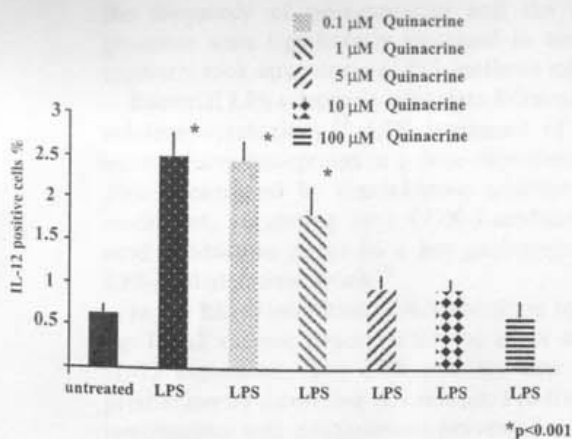


Fig. 2. Quinacrine treatment inhibits LPS-induced IL-12 production in a concentration-dependent fashion. The bars represent the mean  $\pm$  S.E.M. of 10 individual determinations. \*Significantly different from the control (untreated) at  $P < 0.001$ .

instead of an anti-PIBF antibody. Neutralization of endogenously produced PIBF by anti-PIBF antibody resulted in a more than threefold increase in the percentage of IL-12 positive mononuclear cells ( $P < 0.001$ ) (Fig. 3), while an irrelevant antibody of the same isotype did not alter IL-12 expression.

Quinacrine completely abrogated the effect of anti-PIBF treatment on IL-12 production (Fig. 3), suggesting that PLA2 block by PIBF is involved in inhibition of IL-12 synthesis.

## DISCUSSION

Successful allopregnancy is a Th2 phenomenon<sup>13</sup> and a failure of the generation of Th2-type cytokine responses is associated with recurrent abortions, complications and poor pregnancy outcome.<sup>14</sup>

IL-12 induces interferon (IFN)- $\gamma$  production and enhances NK-cell cytotoxicity.<sup>15</sup> Normal human pregnancy is characterized by low peripheral NK activity.<sup>16</sup> There is evidence for the relationship between increased NK activity and spontaneous abortions of unknown etiology.<sup>17</sup> Cytotoxic mechanisms and cytokines produced by NK cells – such as tumor necrosis factor (TNF- $\alpha$ ), via facilitating PG synthesis – can induce ablation of placenta, uterine contractions and initiate the induction of labor.

Several data suggest that PLA2 activation is a necessary signal in human NK cytotoxicity.<sup>18</sup> PLA2 releases fatty acid from the sn-2 position of phospholipids. This reaction is of particular importance in inflammation as it liberates AA, a rate-limiting precursor of potent inflammatory mediators, PGs and leukotrienes. The anti-inflammatory effects of quinacrine

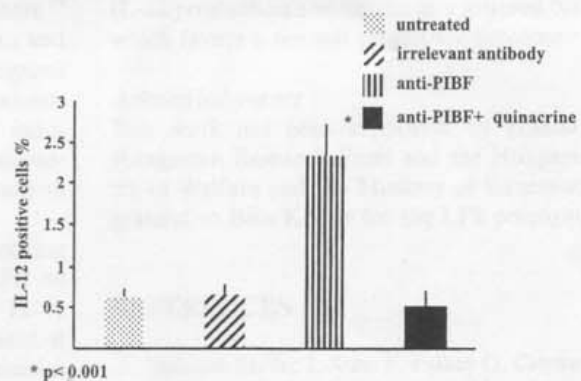


Fig. 3. The effect of anti-PIBF and simultaneous quinacrine treatment on IL-12 production. The bars represent the mean  $\pm$  S.E.M. of 20 determinations. \*Significantly different from the control (untreated) at  $P < 0.001$ .



have been proposed to be associated with an inhibitory effect on PLA2, as its immunological effect was overcome by supplying exogenous AA as a substrate for the cyclooxygenase and lipoxygenase pathway. Quinacrine inhibited the generation of cytotoxic T lymphocytes from human peripheral blood T cells. Treatment of human NK cells with PLA2 inhibitor diminishes their ability to lyse K562 target cells.<sup>18</sup>

There is evidence that PGs, in particular PGE2 and PGF2 $\alpha$ , are important mediators in the onset of human labor. Fetal membrane PLA2 activity increases throughout gestation. Total cellular cPLA2 has also been found to be high before the onset of labor and there is evidence for increased amnion COX-2 enzyme activity and mRNA expression at term.<sup>19,20</sup> Inflammatory mediators rapidly induce cPLA2 and COX-2 activity, with a parallel increase in PGE2 synthesis in amnion and decidual cells.<sup>21,22</sup>

Hilkens et al. have shown that the net modulatory effect of PGE2 on the cytokine secretion profile of T cells critically depends on the mode of T cell activation and consequently the availability of IL-2.<sup>23</sup> Ching et al. demonstrated a positive correlation between NK cell cytotoxicity and the plasma PG F series concentration in cord blood.<sup>24</sup> Mice with ablated gene for the PGF2 $\alpha$  receptor experience normal gestation, but fail to initiate labor and delivery.<sup>25</sup> Our earlier studies revealed an increased PGF2 $\alpha$  sensitivity of human peripheral lymphocytes together with a decreased PGE2 sensitivity during labor.<sup>26</sup>

Selective COX-2 inhibitors have been shown to block spontaneous uterine contractions in the rat and prevent pre-term birth in humans. Acetylsalicylic acid (ASA) treatment starting before implantation may reduce the rate of abortion in some types of patients suffering from recurrent miscarriages.<sup>27</sup> Furthermore, the frequency of post-maturity and the length of gestation were significantly increased in women who regularly took large doses of PG synthesis inhibitors.<sup>28</sup>

Bacterial LPS selectively stimulate PG synthesis and enhance cytotoxicity.<sup>11</sup> LPS treatment of pregnant mice induces resorptions in a dose-dependent manner. This is corrected by simultaneous addition of indomethacin, suggesting that COX-2-mediated eicosanoid production might be a key pathologic event in LPS-mediated fetal death.<sup>29</sup>

In our hands inhibition of AA liberation by blocking the PLA2 enzyme counteracted the effect of LPS on IL-12 expression, thus LPS possibly acts on IL-12 production by increasing AA release. Pre-treatment of lymphocytes with progesterone prevented the stimulating effect of LPS on IL-12 production.

Earlier we showed that PIBF produced by progesterone treated pregnancy lymphocytes affects AA metabolism of lymphoid cells<sup>1</sup> and the subsequent

decrease in AA release runs parallel with lower cytotoxic activity.<sup>18</sup> Our previous data indicate that PIBF may induce a Th2 type cytokine response.<sup>7</sup> The present data show that neutralization of endogenously produced PIBF by an anti-PIBF antibody results in a more than threefold increase in the percentage of IL-12 positive mononuclear cells, which is corrected by simultaneous quinacrine treatment, suggesting that PIBF inhibits IL-12 production via an action on AA metabolism.

Prostaglandin synthesis inhibitors were reported to be effective in the treatment of threatened abortion and pre-term labor in humans. As a site of action, the uterine smooth muscle has generally been accepted. The present data suggest that immunological actions of PG synthesis inhibitors provide additional benefit to their known effects on the uterine musculature and blood supply.

Earlier we reported that progesterone-binding capacity in lymphocytes of patients at risk for premature pregnancy termination is impaired in comparison with those from healthy pregnant women.<sup>30</sup> Lymphocytes from the former patients are unable to bind a sufficient amount of progesterone, therefore no PIBF is released, and thus AA metabolism proceeds normally and results in high IL-12 production and consequently higher cytotoxic activity. This concept is supported by our clinical observation,<sup>31</sup> which revealed a beneficial effect of low dose aspirin treatment of recurrent aborters, selected on the basis of repeatedly high NK activity and reduced PIBF-producing capacity of their lymphocytes. Aspirin treatment reduced NK activity and resulted in an 82% success rate in contrast to the untreated group, where the success rate was 44%.

Based on these findings we suggest the following mechanism: progesterone binding of the lymphocytes is followed by the release of PIBF that inhibits AA release. The subsequent block of PG synthesis reduces IL-12 production and results in a lowered NK activity, which favors a normal pregnancy outcome.

#### Acknowledgments

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## ..... **Progesterone-Dependent Immunomodulation**

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### **Abstract**

The biological effects of progesterone are mediated by a 34-kDa protein named the progesterone-induced blocking factor (PIBF). PIBF, synthesized by lymphocytes of healthy pregnant women in the presence of progesterone, inhibits arachidonic acid release as well as NK activity, and modifies the cytokine balance. Within the cell the full-length PIBF is associated with the centrosome, while secretion of shorter forms is induced by activation of the cell. PIBF induces nuclear translocation of STAT6 as well as PKC phosphorylation and exerts a negative effect on STAT4 phosphorylation. The concentration of PIBF in pregnancy urine is related to the positive or negative outcome of pregnancy; furthermore, premature pregnancy termination is predictable by lower than normal pregnancy PIBF values. In vivo data suggest the biological importance of the above findings. Treatment of pregnant Balb/c mice with the antiprogesterone RU 486 results in an increased resorption rate, which is associated with the inability of spleen cells to produce PIBF. High resorption rates induced by progesterone receptor block as well as those due to high NK activity are corrected by simultaneous PIBF treatment.

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### **Progesterone-Induced Blocking Factor Mediates the Immunological Effects of Progesterone**

Beside its well-known endocrine effects, progesterone is endowed with immunomodulatory properties, which contribute to its pregnancy-protective role. High concentrations of progesterone prolong the survival of xenogenic and allogeneic grafts [1, 2], and the hormone affects various phases of the immune response in vitro [3–5].

NK activity of lymphocytes of healthy pregnant women can be suppressed by a relatively low (100–400 nM) concentration of progesterone, whereas 100 times higher concentrations are required for reducing the natural cytotoxic activity of nonpregnancy lymphocytes [6] and this effect is inhibited by equimolar concentrations of RU 486 (a blocker of progesterone and glucocorticoid receptors) [7].

The biological effects of progesterone are mediated by a 34-kDa protein, named the progesterone-induced blocking factor (PIBF). PIBF, synthesized by lymphocytes of healthy pregnant women in the presence of progesterone [8, 9], inhibits arachidonic acid release by acting directly on the phospholipase A2 enzyme [10] as well as NK activity and modifies the cytokine balance [11]. Through the above mechanisms PIBF exerts an antiabortive effect [12–14].

### **Molecular Structure of PIBF**

The PIBF cDNA encodes a protein of 757-amino acid residues with an 89-kDa predicted molecular mass, which shows no significant amino acid sequence homology with any of the known proteins [15].

The full-length PIBF is associated with the centrosome, while secretion of shorter forms, among others; the previously described secreted 34-kDa protein is induced by activation of the cell. The 48-kDa N-terminal part of PIBF is biologically active, and the region responsible for modulating NK activity is encoded by exons 2–4 [15]. These data suggest that PIBF might act both as a transcription factor and as a cytokine, via binding to receptors.

### **Biological Effects of PIBF**

PIBF affects arachidonic acid metabolism of lymphoid cells and the subsequent decrease in prostaglandin and/or leukotriene synthesis goes in parallel with lower cytotoxic activity [8]. IL-12 induces NK activity and there is evidence for a relationship between high NK activity and pregnancy termination both in mice [16, 17] and humans [18]. In our hands, neutralization of PIBF resulted in an increased IL-12 expression, which was corrected by treatment of the cells with phospholipase A2 inhibitor [10].

These results suggest that PIBF inhibits arachidonic acid release. The subsequent block of prostaglandin synthesis reduces IL-12 production and results in a lowered cytotoxic NK activity, which favors a normal pregnancy outcome. In line with this hypothesis, aspirin treatment starting before implantation may

reduce the rate of abortion in patients suffering from recurrent miscarriages [19]. Furthermore, the frequency of postmaturity and the length of gestation were significantly increased in women who regularly took large doses of prostaglandin synthesis inhibitors [20].

### Cytokine Effects and Signal Transduction

The effect of PIBF on NK activity is manifested via an altered cytokine production both *in vitro* and *in vivo*. Neutralization of endogenous PIBF in pregnancy lymphocytes by a PIBF-specific antibody results in increased NK activity, which is corrected by IL-12-neutralizing antibody [11]. PIBF inhibits IL-12 synthesis by activated lymphocytes, and recent data from our laboratory revealed an increased IL-12 production by peripheral lymphocytes of women with pathological pregnancies and high NK activity [21]. *In vitro* PIBF treatment of activated lymphocytes favors the production of Th2 type of cytokines [22]. Joachim et al. [23] detected reduced PIBF concentrations, together with increased resorption rates in pregnant mice that had been subjected to acoustic stress. Both PIBF levels and resorption rates were corrected by treating the animals with a retroprogesterone, and this was accompanied by a significantly increased decidual IL-4 production. These data together support the concept that the NK inhibitory action of PIBF is mediated – at least in part – by cytokines, and PIBF induces a Th2-biased cytokine production.

STAT transcription factors mediate virtually all cytokine-driven signaling, whereas protein kinase C (PKC) plays a critical role in the differentiation of T cells to the Th1 or Th2 type.

STAT6 and STAT4 specifically mediate signals that stem from IL-4 and IL-12 receptors, respectively [24]. INF- $\gamma$  has been shown to be a negative regulator of STAT6-dependent transcription of target genes [25]. STAT4 is mainly phosphorylated by the IL-12-mediated signaling pathway in T cells and in NK cells by the tyrosine kinases Jak2 and Tyk2 [26, 27]. Mice lacking STAT4 clearly demonstrated that STAT4 is necessary for the generation of Th1 cells [28, 29].

STAT6-deficient animals are unable to mount an immune response to helminthic parasites and therefore are unable to clear the parasitic infections [30]. IL-4 signaling via STAT6 appears to play a role in the development of allergic asthma; it has been also observed that STAT6-deficient mice did not develop airway hyperresponsiveness after allergen sensitization like their wild-type littermates and were protected from allergic asthma [31].

The 48-kDa recombinant human PIBF as well as two smaller proteins encoded by exons 2–4 and 13–16 induce nuclear translocation of STAT6 [32].

PIBF exerts a negative effect on STAT4 phosphorylation and inhibits IL-12-induced STAT4 activation.

The PKC pathway represents a major signal transduction system that is activated following ligand stimulation of receptors by hormones, neurotransmitters, and growth factors. PKC (80-kDa proteins) play a critical role in the regulation of differentiation and proliferation in many cell types and in the response to diverse stimuli [33].

Development of naive T cells into type 1 (Th1) or type 2 (Th2) effector cells is thought to be under the control of cytokines. IL-12 and IL-4 are widely accepted to be the major factors inducing T cells to develop into type 1 or type 2 cells [34]. When IL-12 and IL-4 are present, murine and human T cell differentiation is regulated by the balance of PKC and calcium signaling within T cells [35].

It has long been known that Th2 clones show reduced calcium flux after activation compared with Th1 clones [36]. High levels of PKC activity combined with low calcium signals favor Th2 development, while predominance of calcium signaling with low PKC activity favors Th1 development [35]. Signals downstream of PKC and calcineurin directly result in preferential type 1 or type 2 cytokine gene expressions, perhaps via expression of transcription factors associated with Th2 cells [37, 38].

Phosphorylation of PKC is increased in the cytoplasmic fraction of lymphocytes treated with the 48-kDa recombinant PIBF as well as with a peptide encoded by exons 13–16. Intracellular calcium levels are not altered by PIBF treatment. High PKC activity and low intracellular calcium levels favor the development of Th2 cytokine-sensitive cells, whereas inhibition of STAT4 phosphorylation decreases the sensitivity of the cell to Th1 cytokines. These together might account for the Th2-biased immune response induced by PIBF [32].

### PIBF Concentration in Pregnancy Urine Is Related to the Outcome of Pregnancy

PIBF is a secreted molecule; thus it might appear in biological fluids, and due to its small molecular weight, it is filtrated into the urine. Urinary PIBF concentrations of 86 healthy nonpregnant individuals and those from 496 pregnant women were determined by ELISA. The concentration of PIBF continuously increased until the 37th gestational week of normal pregnancies, followed by a sharp decrease after the 41st week of gestation. In pathological pregnancies urinary PIBF levels failed to increase. Samples from 86 healthy nonpregnant individuals were used for determining the threshold of nonpregnancy values. Eighty percent of women with a normal, uneventful pregnancy whereas only 10% of

those whose pregnancies ended up in miscarriage, or preterm labor had higher PIBF concentrations than control threshold. The sensitivity (defined as the ability to correctly identify those who will deliver preterm) and specificity (the ability to correctly identify those who will not deliver preterm) of the test for predicting pregnancy failure are 90 and 80%, respectively. These data suggest that low PIBF values might indicate the onset of spontaneous pregnancy termination.

Women with toxemia had lower PIBF values than healthy pregnant women. Since PIBF favors a Th2 cytokine response, these women should have a relative Th1 dominance. Rein et al. [39] reported that trophoblast cells from preeclamptic women produce significantly less IL-10 in the 3rd trimester of pregnancy than those from healthy pregnant women, and an excessive Th1 activity has been associated with toxemia [40]. Several studies have shown that the clinical severity of preeclampsia is related to the severity of cytokine abnormalities [41]. PIBF concentrations in urine of toxemic women were related to the clinical symptoms. PIBF levels of women demonstrating hypertension only did not differ from those of healthy pregnant women. In contrast to this, only 33% of women with two or more symptoms had levels higher than the threshold. This is in line with earlier observations of Varga et al. [42], who could not demonstrate an increased peripheral NK activity in the group of preeclamptic patients with a single symptom (hypertension), whereas lymphocytes of preeclamptic women with at least two symptoms showed a significantly increased NK activity. PIBF inhibits NK activity both in vitro [2, 5], and the lack of PIBF results in an increased NK activity [11].

All the women bearing small-for-date babies had lower than normal PIBF values. This suggests that similar mechanisms might play a role in the development of intrauterine growth retardation. Bartha et al. [43] have shown an association between increased TNF- $\alpha$  levels and intrauterine growth retardation. In our hands treatment of pregnant mice with high NK activity spleen cells resulted in elevated serum and placental TNF- $\alpha$  levels in pregnant Balb/c mice, together with increased resorption rates. Simultaneous TNF- $\alpha$  administration corrected both TNF- $\alpha$  levels and resorption rates. In vitro data suggest that PIBF counteracts the cytotoxic action of TNF- $\alpha$  but does not interfere with its production [44].

In general, the concentration of PIBF is related to the positive or negative outcome of pregnancy; furthermore, premature pregnancy termination is predictable by lower than normal pregnancy PIBF values.

#### PIBF Exerts an Antiabortive Effect in Mice

In vivo data suggest the biological importance of the above findings. Treatment of pregnant Balb/c mice with the antiprogesterone RU 486 results in

an increased resorption rate, which is associated with the inability of spleen cells to produce PIBF. High resorption rates induced by progesterone receptor block as well as those due to high NK activity are corrected by simultaneous PIBF treatment [13, 14].

Neutralization of endogenous PIBF by a PIBF-specific antibody terminates pregnancy in mice [11]. Depletion of NK activity with anti-NK antibodies counteracts the above effects [45]. Both anti-PIBF treatment and that with progesterone receptor blocker result in increased splenic NK activity, together with reduced IL-10 and an increased IFN- $\gamma$  production of the spleen cells.

Based on these data we suggest that the immunological pregnancy-protective effects of progesterone are manifested via the following mechanism: in the presence of progesterone activated pregnancy lymphocytes synthesize a mediator (PIBF), which, by interfering with arachidonic acid metabolism and by inducing a Th2-biased immune response, allows pregnancy to go to term.

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# The Progesterone-Induced Blocking Factor Modulates the Balance of PKC and Intracellular Ca<sup>++</sup>

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## Keywords

Cytokine, signaling, Th2

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## Problem

Progesterone-induced blocking factor (PIBF) induces Th2 biased cytokine production; therefore, this study investigates the effects of PIBF on the protein kinase C (PKC)/Ca<sup>++</sup> system – which plays a key role in Th1/Th2 differentiation.

## Method of study

Proteins from PIBF-treated cells were reacted on Western blots with phospho-specific antibodies recognizing different PKC isoforms. Intracellular free calcium was measured by flow cytometry.

## Results

Both interleukin (IL)-4 and PIBF induced PKC phosphorylation, which was abrogated by anti-IL-4R $\alpha$  or anti-PIBF immunoglobulin G pre-treatment. PIBF treatment did not alter intracellular Ca<sup>++</sup> levels. Inhibition of PKC $\zeta$  or PKC $\theta$  phosphorylation, but not that of PKC $\alpha/\beta$  resulted in the loss of STAT6 and Jak1 phosphorylation by PIBF.

## Conclusions

Our data show that PIBF phosphorylates PKC via binding to the IL-4R, without affecting intracellular Ca<sup>++</sup>. Phosphorylation of PKC $\zeta$  and PKC $\theta$  is required for Jak1 and STAT6 activation, whereas PKC $\alpha/\beta$  is not involved. These findings explain the mechanism by which PIBF supports a Th2 dominant cytokine pattern.


## Introduction

The protein kinase C (PKC) pathway represents a major signal transduction system that is activated following ligand-stimulation of receptors by hormones, neurotransmitters, and growth factors. PKC plays a critical role in the regulation of differentiation and proliferation in many cell types as well as in the response to diverse stimuli. To date, 11 PKC isoenzymes have been described and classified into three subfamilies:

Conventional, Ca<sup>++</sup> dependent protein kinase C (cPKC) isoforms;  $\alpha, \beta, \gamma$ , or novel Ca<sup>++</sup> independent

protein kinase C (nPKC) isoforms;  $\delta, \epsilon, \eta, \theta, \mu$ , and atypical, phospholipase- and Ca<sup>++</sup> independent protein kinase C (aPKC) isoforms;  $\xi, \iota, \lambda$ .<sup>1</sup>

Development of naive T cells into type 1 (Th1) or type 2 (Th2) effector cells is thought to be under the control of cytokines. Interleukin (IL)-12 and IL-4 are widely accepted to be the major factors inducing T cells to develop into type 1 or type 2 cells.<sup>2</sup> When IL-12 and IL-4 are present, murine and human T-cell differentiation is regulated by the balance of PKC and calcium signaling within T cells.<sup>3</sup> High levels of PKC activity combined with low calcium signals favor Th2 development, while predominance

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of calcium signaling with low PKC activity favors Th1 development.<sup>3</sup> Signals downstream of PKC and calcineurin via activation of transcription factors directly result in preferential type 1 or type 2 cytokine gene expression.<sup>4,5</sup>

Progesterone favors a Th2 dominant cytokine pattern<sup>6</sup> and our previous data show that *in vitro* treatment of activated lymphocytes with a progesterone-induced protein [progesterone-induced blocking factor (PIBF)] leads to increased production of Th2 type of cytokines.<sup>7</sup> Lymphocytes from pregnant women at risk for premature pregnancy termination produce increased levels of IL-12 together with low levels of PIBF and IL-10.<sup>8</sup> Furthermore, neutralization of endogenous PIBF activity in pregnant mice with anti-PIBF immunoglobulin (Ig) G, results in increased IFN $\gamma$  production.<sup>9</sup> As PIBF exerts its biological effects by inducing a Th2 dominant cytokine production, this study was aimed at investigating the effects of PIBF on the PKC/Ca<sup>++</sup> signal transduction pathways.

## Materials and methods

### Recombinant Human PIBF and Antibodies

The 48-kDa N-terminal recombinant human PIBF was prepared as described earlier.<sup>10</sup>

Polyclonal anti-PIBF antibodies were generated by immunizing rabbits with the 48-kDa N-terminal recombinant human PIBF. IgG was affinity purified on protein A columns.

Monoclonal anti-human IL-4 receptor  $\alpha$  (IL-4R $\alpha$ ) antibody (from R&D Systems Europe Ltd, Abingdon, UK) was used for treating lymphocytes. Mouse IgG2A specific Ig (from Sigma-Aldrich Inc., Budapest, Hungary), and anti rabbit IgG (from Dako, Glostrup, Denmark) were used as isotype controls. Rabbit polyclonal phospho-specific (Thr-410/403) anti-human PKC $\zeta$ , phospho-specific (Thr-538) anti human PKC $\theta$ , and phospho-specific (Thr-638/641) anti-human PKC $\alpha/\beta$  II (all from Cell Signaling Technology, Beverly, MA, USA) were used for blocking and detection of PKC isotypes. Rabbit polyclonal phospho-specific (Tyr-641) anti-human STAT6, rabbit polyclonal phospho-specific (Tyr-1022/Tyr-1023) anti-human Jak1 and HRPO-labeled anti-rabbit IgG (Dako) were used for Western blotting. Polyclonal rabbit anti-human STAT6, anti-human Jak1 (both from Santa Cruz Biotechnology Inc. CA, USA) and rabbit anti-human  $\beta$  actin

(Sigma-Aldrich Inc.) were used for controlling the loading on Western blots.

### Treatment of Lymphocytes

Lymphocytes of healthy non-pregnant volunteers were isolated from heparinized venous blood on Ficoll–Paque gradient (Pharmacia, Uppsala, Sweden), and washed in RPMI 1640 medium (Gibco BRL, Life Technologies, Paisly, Scotland).

The cells were treated with 1  $\mu$ g/mL of Phytohemagglutinin (Sigma-Aldrich Inc.) for 24 hr and then incubated at 37°C in 5% CO<sub>2</sub> with the following:

- RPMI medium 1640 (control).
- 48-kDa N-terminal recombinant human PIBF.
- Recombinant human IL-4 (from R&D Systems Europe Ltd. Abingdon, UK).
- 48-kDa N-terminal recombinant human PIBF together with anti-PIBF antibody or with anti-human IL-4R $\alpha$  antibody, or with phospho-specific anti-human PKC $\zeta$ , or with phospho-specific PKC $\theta$ , or with phospho-specific anti-human PKC $\alpha/\beta$  II.
- Recombinant human IL-4 together with monoclonal anti-human IL-4R $\alpha$  antibody, or with phospho-specific anti-human PKC $\zeta$ , or with phospho-specific PKC $\theta$ , or with phospho-specific anti-human PKC $\alpha/\beta$  II.

To determine the specificity of the reactions, lymphocytes were treated with the above antibodies without PIBF. An N-terminal recombinant human PIBF (48-kDa) and the cytokines were used at a concentration of 200 ng/mL for  $5 \times 10^7$  cells, anti-human IL-4R $\alpha$  and anti-human PIBF antibodies were applied in a blocking concentration of 50  $\mu$ g/mL for  $5 \times 10^7$  cells, and the phospho-specific PKC antibodies were diluted 1:100 for the treatments.

All treatments were performed for 20 min. Iso-type controls were used with each treatment. For controlling the effect of possible lipopolysaccharide contamination of recombinant PIBF, parallel lymphocyte samples were treated with lysates of *Escherichia coli* that had undergone the same purification procedure as the recombinant PIBF. Lysate of *E. coli* was used in a concentration of 200 ng/mL for  $5 \times 10^7$  cells.

Pre-treatment of lymphocytes with the PKC inhibitor, Staurosporine (Sigma Aldrich Inc.) was performed at a concentration of 400 ng/mL for  $5 \times 10^7$  cells at 37°C in 5% CO<sub>2</sub> for an hour.

### Western Blotting

Following the treatment, lymphocytes were washed in PBS and then in PBS containing 1 mM sodium-vanadate and 5 mM NaF by centrifugation (13,000 rpm for 40 s). Pellets were resuspended in 3× volumes of buffer containing 20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM sodium-vanadate, 1 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycoltetraacetic acid, 5 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride, aprotinin (0.01 µg/mL), leupeptin (0.025 µg/mL), 400 mM NaCl, 20% glycerol. Samples were frozen and thawed three times, and kept on ice for a further 20 min. After centrifugation at 15,000 rpm at 4°C for 30 min, the supernatants were collected, and the protein content of the extracts was determined. For detection of different transcription factors in the extracts 25 µg of total protein was separated by 7.5% SDS-PAGE, and transferred to a Hybond ECL membrane (Amersham Biosciences, Little Chalfont, UK) overnight at 54 mA. The blots were blocked with 3% non-fat dry milk in TBS-Tween solution for an hour, then incubated with 1:1000 diluted rabbit polyclonal phospho-specific anti-human PKC $\zeta$  or phospho-specific anti human PKC $\theta$  or phospho-specific anti-human PKC $\alpha/\beta$  II for 4 hr or with 1:20,000 diluted rabbit polyclonal phospho-specific anti-human STAT6, or with 1:2000 diluted rabbit polyclonal phospho-specific anti-human Jak1 for 1 hr in TBS-Tween solution containing 3% non-fat dry milk. For controlling the loading 1:1000 diluted rabbit polyclonal anti-human STAT6 or 1:1000 diluted rabbit polyclonal anti-human Jak1 antibodies or 1:1000 diluted rabbit anti-human  $\beta$  actin antibodies were used. After three washing cycles (10 min each in TBS-Tween pH 7.4), the blots were incubated at room temperature for 45 min with 1:2000 diluted HRP0-labeled anti-rabbit IgG, and then washed six times (10 min each) in TBS-Tween. Antibody binding was detected using an ECL kit (Perkin Elmer Life Sciences, Boston, MA, USA) according to the manufacturer's instructions.

### Flow Cytometric Determination of Intracellular $\text{Ca}^{++}$

In peripheral lymphocytes from healthy non-pregnant volunteers intracellular free calcium was measured using Fluo-3 AM according to the protocol described by Minta et al.<sup>11,12</sup>

Briefly,  $10^6$  cells were incubated for 30 min at room temperature in 100 µL tissue culture medium containing 10 µM Fluo-3 AM. The cell suspensions were diluted with 10 mL RPMI containing 10% FCS and incubated for further 30 min. Samples were washed twice in RPMI containing 10% FCS. Activation was carried out with ionomycin (1 µg/ $10^6$  cells) as a non-specific activator<sup>13,14</sup> or 48-kDa N-terminal recombinant human PIBF (5 µg/ $10^6$  and 20 µg/ $10^6$  cells). 7-Aminoactinomycin D (7-AAD) labeling was used<sup>15</sup> to distinguish dead cells by their higher fluorescence at 647 nm (FL3 channel). The viable cells were gated and all further calculations were performed excluding the 7-AAD positive cells. Increase of fluorescence was measured in a Becton Dickinson FACS Calibur flow cytometer using the CellQuest program. The mean fluorescence intensity of Fluo-3 AM dye (proportional to the cytosolic calcium level) was determined at 526 nm (FL1 channel). After measuring basal fluorescence at 526 nm, the activating agent was added and the measurement continued for further 100–400 s, enabling us to follow the alterations of intracellular free calcium level (FL1 fluorescence intensity) in function of time. Gates were created along the time axis of the activation dot plots at definite time points and the mean fluorescence intensity at 526 nm was statistically analyzed from each gate.

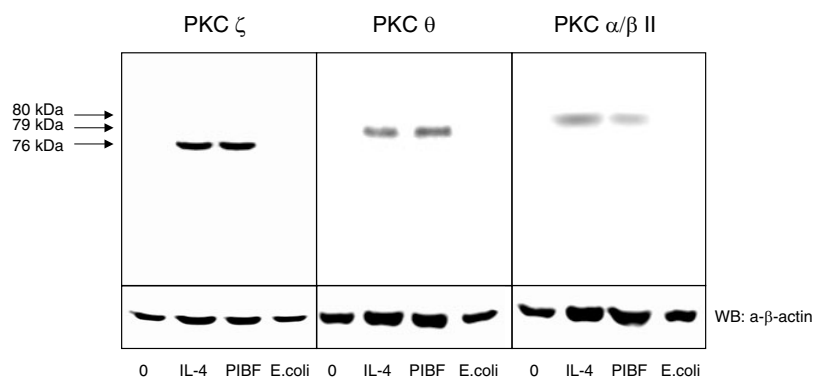
## Results

### PIBF Induces PKC Phosphorylation Without Affecting Intracellular $\text{Ca}^{++}$ Levels

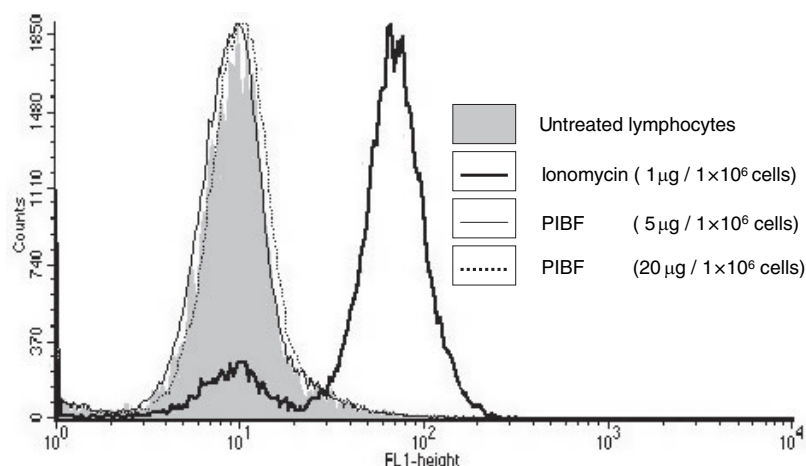
In order to investigate the possible involvement of the PKC/ $\text{Ca}^{++}$  signaling pathway in cytokine effects of PIBF, we tested the impact of PIBF treatment on PKC phosphorylation.

Following incubation of lymphocytes with 48-kDa N-terminal recombinant human PIBF or recombinant human IL-4 the cells were lysed, proteins were separated on SDS-PAGE, blotted to nitrocellulose and reacted with phospho-specific antibodies recognizing the PKC isoforms; PKC $\alpha/\beta$  II, PKC $\theta$  and PKC $\zeta$ . Both IL-4 and PIBF treatment resulted in phosphorylation of all three PKC isoforms (Fig. 1).

Intracellular free calcium in PIBF-treated peripheral lymphocytes was determined by flow cytometry. Lymphocytes treated with the non-specific activator ionomycin served as positive controls. Fig. 2 shows that PIBF-treatment did not alter intracellular  $\text{Ca}^{++}$  levels.



**Fig. 1** The effect of progesterone-induced blocking factor (PIBF) on phosphorylation of protein kinase C (PKC) isoforms. Proteins from lysates of PIBF-treated cells were separated on SDS-PAGE, blotted to nitrocellulose membranes and reacted with phospho-specific antibodies recognizing the PKC isoforms; PKC $\zeta$ , PKC $\theta$  and PKC $\alpha/\beta$  II. Controls include the lysates of untreated cells, and since the recombinant PIBF was produced by *Escherichia coli*, lysate of the bacterium which had been treated the same way as recombinant PIBF during purification.



**Fig. 2** The effect of progesterone-induced blocking factor (PIBF) on intracellular  $\text{Ca}^{2+}$  levels. Intracellular calcium levels were determined in PIBF-treated cells by flow cytometry. Ionomycin treated cells were used as positive controls.

### PIBF Phosphorylates PKC via Binding to the IL-4 Receptor $\alpha$ Chain

Since earlier data suggest that PIBF uses the IL-4R $\alpha$  chain for STAT6 signaling, we tested whether functional IL-4R $\alpha$  was required for PKC activation. The effect of PIBF on the phosphorylation of the three PKC isoforms was abrogated by pre-treatment of the cells with blocking concentrations of anti IL-4R $\alpha$  antibody or anti-PIBF IgG (Fig. 3).

### Inhibition of PKC Phosphorylation Counteracts the Effects of PIBF on Jak1 and STAT6

Recent evidence suggests that PKC $\zeta$  activity is needed for Jak1 function.<sup>16</sup> Therefore we investigated the requirement of PKC phosphorylation for Jak1

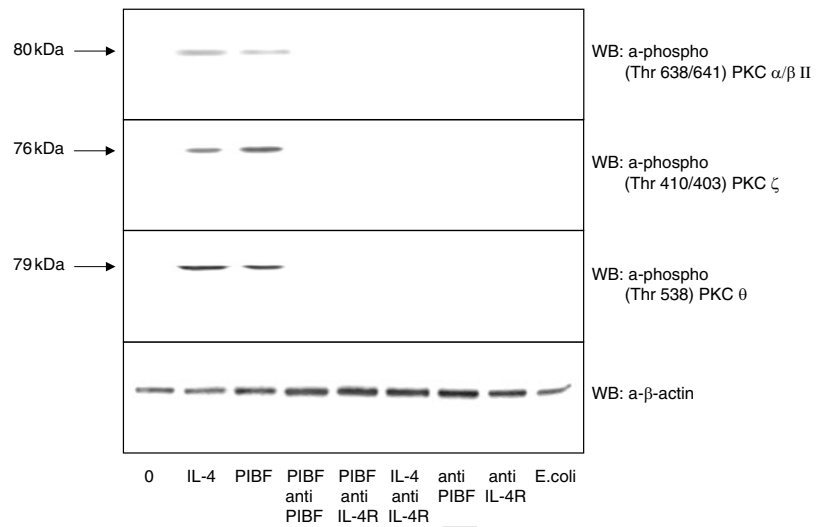
and STAT6 activation by PIBF. Lymphocytes were treated with PIBF in the presence of the PKC inhibitor Staurosporine and the cell lysates were reacted on a Western blot with anti-phospho-STAT6 or anti-phospho-Jak1 antibodies. Inhibition of PKC activity abrogated PIBF-induced Jak1- and STAT6 phosphorylation (Fig. 4). The use of PKC isotype specific antibodies in place of the PKC inhibitor revealed that blocking PKC $\zeta$ , and PKC $\theta$  with specific antibodies, interfered with PIBF-induced Jak1- and STAT6 phosphorylation. Blocking of PKC $\alpha/\beta$  had no effect (Fig. 5).

### Discussion

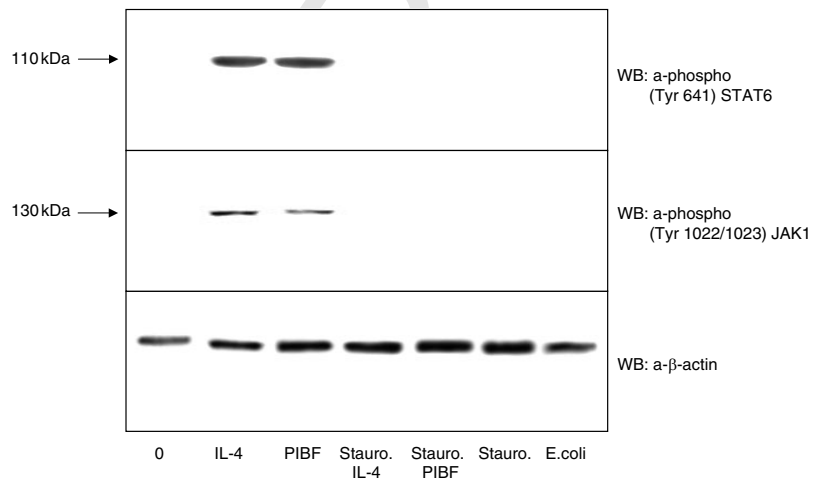
In this paper, we show that the PIBF – which induces a Th2 dominant cytokine pattern and exerts a



**Fig. 3** Blocking of the interleukin (IL)-4R $\alpha$  inhibits progesterone-induced blocking factor (PIBF)-induced protein kinase C (PKC) phosphorylation. Lymphocytes were treated with PIBF in the presence of a monoclonal anti-IL-4R $\alpha$  antibody. The cells were lysed and the proteins were separated on SDS-PAGE, blotted to nitrocellulose and reacted with phospho-specific antibodies recognizing the PKC isoforms; PKC $\alpha/\beta$  II, PKC $\zeta$  and PKC $\theta$ . Controls include the lysates of untreated cells and, lysates of *Escherichia coli*.



**Fig. 4** Inhibition of protein kinase C (PKC) activity prevents progesterone-induced blocking factor (PIBF)-induced phosphorylation of STAT6 and Jak1. Lymphocytes were treated with PIBF in the presence of the PKC inhibitor Staurosporine (Stauro) and the cell lysates were reacted on a Western blot with anti-phospho STAT6 or anti-phospho Jak1 antibodies.



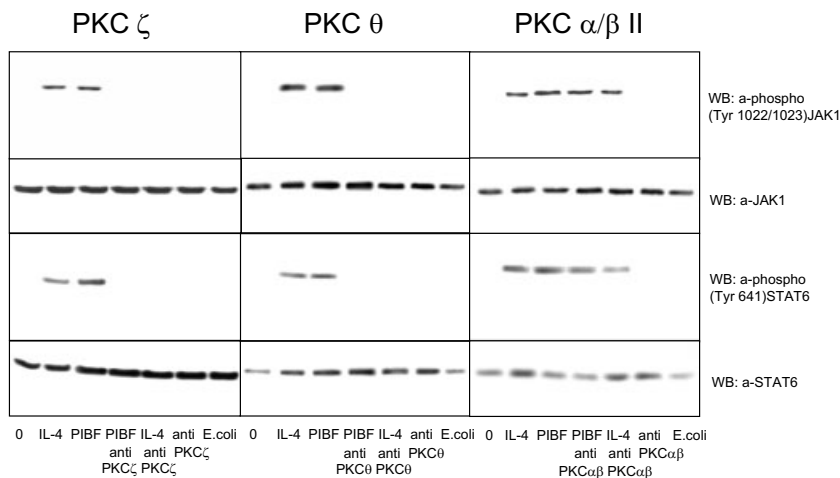
pregnancy protective effect in mice – activates the PKC signal transduction pathway. The Ca<sup>++</sup> independent, novel PKC isoform PKC $\theta$  and the atypical PKC isoform  $\zeta$  are involved the Th2 biased immune response induced by PIBF.

Progesterone-induced blocking factor treatment results in increased Th2 cytokine production both *in vitro*<sup>7</sup> and *in vivo*,<sup>17</sup> and recent data suggest that the above effects are mediated by the Jak/STAT signal transduction pathway (N. Kozma et al. unpublished 6data).

The balance of PKC activity versus calcium signaling is thought to be important in type1/type2 differentiation. Th1 and Th2 T lymphocyte subsets have

been shown to utilize different signaling mechanisms. Th1 activation is characterized by increased intracellular Ca<sup>++</sup> concentrations and low PKC signal, while little or no elevation of intracellular free calcium concentration occurs following activation of Th2 cells via TCR complex compared with Th1 cells.<sup>4,18</sup> In line with the finding, that progesterone does not affect lymphocyte Ca<sup>++</sup> channels<sup>18</sup> – PIBF induced PKC phosphorylation, without any effect on intracellular Ca<sup>++</sup> levels.

T cells express multiple isotypes of PKC, and though it is accepted that PKCs play a role in T-cell activation, little is known about the function of individual PKC isotypes.



**Fig. 5** The effect of blocking protein kinase C (PKC) activity by isoform specific antibodies on progesterone-induced blocking factor (PIBF)-induced Jak1 and STAT6 phosphorylation. PIBF-treated cells were incubated with blocking concentrations of PKC $\zeta$ , PKC $\theta$  or PKC $\alpha/\beta$  II. The proteins from the cell lysates were separated on SDS-PAGE, blotted to nitrocellulose and reacted with anti-phospho STAT6 or anti-phospho Jak1 antibodies.

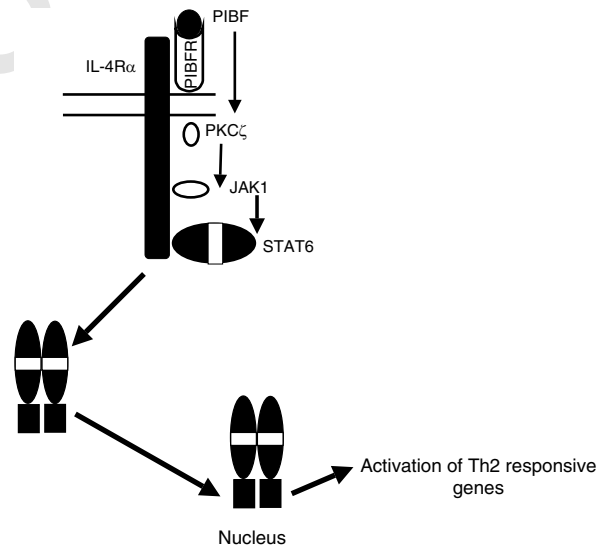
Protein kinase C $\theta$  and PKC $\zeta$  are both involved in the induction of type 2 development.<sup>19,20</sup> PKC $\zeta$  deficiency leads to impaired secretion of Th2 cytokines because of the inability of PKC $\zeta$ <sup>-/-</sup> CD4<sup>+</sup> T cells to differentiate adequately along the Th2 lineage.<sup>16</sup> PKC $\zeta$ <sup>-/-</sup> mice have a defect in Th2 cells but not in naive T-cell proliferation.<sup>21</sup> In case of PKC $\theta$ <sup>-/-</sup> mice, the alterations are not restricted to the Th2 differentiation program,<sup>20–23</sup> implying that the role of PKC $\theta$  has a broader impact in T-cell function.

In this study PIBF phosphorylated PKC $\zeta$ , PKC $\theta$ , and PKC $\alpha/\beta$  in absence, but not in the presence of an anti-IL-4R $\alpha$  antibody, suggesting that a functional IL-4R $\alpha$  is needed for PIBF-induced PKC-activation. Earlier data from our laboratory (N. Kozma et al. unpublished data) show that PIBF signals via the IL-4R and this results in phosphorylation of Jak1 and STAT6.

Protein kinase C $\zeta$  activity is required for Jak1 function. Upon IL-4 signaling PKC $\zeta$  phosphorylates Jak1,<sup>16</sup> and in PKC $\zeta$  deficient cells phosphorylation of STAT6 is dramatically inhibited.<sup>21</sup> In this study we demonstrated increased PKC $\zeta$  phosphorylation, following PIBF treatment, and showed that PKC $\zeta$  and PKC $\theta$  phosphorylation but not that of PKC  $\alpha/\beta$  are required for PIBF-induced Jak1 and STAT6 activation. The first event of PIBF signaling is possibly the phosphorylation of PKC $\zeta$ , which depends on ligation of the IL-4 receptor.<sup>16</sup> Indeed, blocking of the IL-4R $\alpha$  counteracted the PKC activating effect of PIBF. Based on these data we propose the following mechanism; following interaction of PIBF with IL-4R $\alpha$ , PKC $\zeta$  gets phosphorylated and activates Jak1, which in turn phosphorylates STAT6. Intracel-

lular Ca<sup>++</sup> concentrations at the same time remain unchanged.

High PKC activity and low intracellular calcium levels favor the development of Th2 cytokine sensitive cells. These findings with our earlier (unpublished) data – showing STAT6 activation by PIBF – might account for the Th2 biased immune response induced by PIBF (Fig. 6).



**Fig. 6** Protein kinase C (PKC) activation is a required step in progesterone-induced blocking factor (PIBF) signaling to achieve Th2 dominance. Engagement of the PIBF receptor results in PKC phosphorylation. Activated PKC will phosphorylate Jak1, which in turn phosphorylates STAT6. STAT6 dimers are formed, move to the nucleus, where they activate Th2 responsive genes.

## Acknowledgments

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# Progesterone-Induced Blocking Factor Activates STAT6 via Binding to a Novel IL-4 Receptor<sup>1</sup>

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Progesterone-induced blocking factor (PIBF) induces Th2-dominant cytokine production. Western blotting and EMSA revealed phosphorylation as well as nuclear translocation of STAT6 and inhibition of STAT4 phosphorylation in PIBF-treated cells. The silencing of STAT6 by small interfering RNA reduced the cytokine effects. Because the activation of the STAT6 pathway depends on the ligation of IL-4R, we tested the involvement of IL-4R in PIBF-induced STAT6 activation. Although PIBF does not bind to IL-4R, the blocking of the latter with an Ab abolished PIBF-induced STAT6 activation, whereas the blocking of the IL-13R had no effect. PIBF activated suppressor of cytokine signaling-3 and inhibited IL-12-induced suppressor of cytokine signaling-1 activation. The blocking of IL-4R counteracted all the described effects, suggesting that the PIBF receptor interacts with IL-4R  $\alpha$ -chain, allowing PIBF to activate the STAT6 pathway. PIBF did not phosphorylate Jak3, suggesting that the  $\gamma$ -chain is not needed for PIBF signaling. Confocal microscopic analysis revealed a colocalization and at 37°C a cocapping of the FITC PIBF-activated PIBF receptor and PE anti-IL-4R-labeled IL-4R. After the digestion of the cells with phosphatidylinositol-specific phospholipase C, the STAT6-activating effect of PIBF was lost, whereas that of IL-4 remained unaltered. These data suggest the existence of a novel type of IL-4R composed of the IL-4R  $\alpha$ -chain and the GPI-anchored PIBF receptor. *The Journal of Immunology*, 2006, 176: 819–826.

The progesterone-induced blocking factor (PIBF)<sup>3</sup> is coded on locus 13q21-q22, which has been implicated as a common site for somatic deletions in a variety of malignant tumors (1), and the protein has been identified in undifferentiated cells and in malignancies (2). The molecule was initially discovered as a progesterone-induced protein (hence the name), mediating the immunological effects of progesterone (3). PIBF production is a characteristic feature of normal human pregnancy, whereas the lack of PIBF predicts spontaneous pregnancy termination (4). The neutralization of endogenous PIBF results in altered cytokine production and pregnancy termination in mice (5). PIBF induces a Th2-biased cytokine production in vitro (6) and in vivo (7).

STAT proteins were identified as transcription factors mediating virtually all cytokine driven signaling (8–10). STAT4 is needed for generation of Th1 cells (11), whereas STAT6-deficient mice fail to respond to IL-4, suggesting the importance of STAT6 in the activation of IL-4 inducible genes (10).

Suppressor of cytokine signaling (SOCS) proteins negatively regulate the signal transduction of several cytokines (12–14). SOCS-1 and SOCS-3 genes are induced by a variety of cytokines, IL-4 among others (15). CD4<sup>+</sup> naive T cells constitutively express low levels of both SOCS-1 and SOCS-3 mRNA, and the differentiation into the Th1 or Th2 phenotype is accompanied by preferential expression of distinct SOCS mRNA transcripts and proteins (16).

IL-4R is a heterodimeric cytokine receptor system consisting of the signaling high-affinity  $\alpha$ -chain and a transactivating low-affinity chain that can either be the common  $\gamma$ -chain or the IL-13R  $\alpha$ 1-chain (17). The extracellular domain of the IL-4R  $\alpha$ -chain provides nearly the complete ligand-binding affinity of the whole receptor (18). IL-4R  $\alpha$ - and  $\gamma$ -chains associate with the Janus kinases, Jak1 and Jak3, respectively (19). Ligand-induced heterodimerization of IL-4R  $\alpha$ - and  $\gamma$ -chains induces tyrosine phosphorylation of IL-4R $\alpha$  and activation of Jak1 and Jak3 (20). Phosphorylated tyrosine residues of IL-4R $\alpha$  serve as anchor for the Src homology 2 domain of STAT6, which gets phosphorylated (21) and forms homodimers. These translocate to the nucleus and bind to DNA sequences TTCNNN(N)GAA in the promoter region of IL-4 responsive genes (22).

PIBF exerts its biological effects by inducing a Th2-biased cytokine production, therefore, we investigated the effects of PIBF on the STAT6/STAT4 signaling pathways, and because activation of the STAT6 pathway is initiated through IL-4R, the possibility that PIBF signals via IL-4R was investigated.

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<sup>3</sup> Abbreviations used in this paper: PIBF, progesterone-induced blocking factor; SOCS, suppressor of cytokine signaling; PI-PLC, phosphatidylinositol-specific phospholipase C; siRNA, small interfering RNA; SCR, scrambled.



## Materials and Methods

### *Abs and PIBF*

The 48-kDa N-terminal recombinant human PIBF was prepared as described earlier (3) and is referred to as PIBF throughout our report. Polyclonal anti-PIBF Abs were generated by immunizing rabbits with the 48-kDa N-terminal recombinant human PIBF. IgG was affinity purified on protein A columns.

Monoclonal anti-human IL-4R $\alpha$  as well as goat anti-human IL-13R $\alpha$ 1 Abs (all from R&D Systems) were used for treating lymphocytes. Mouse IgG2A-specific Ig (Sigma-Aldrich), and rabbit IgG-specific and goat IgG-specific Abs (both from DakoCytomation) were used as isotype controls. Rabbit polyclonal phospho-specific (Tyr<sup>641</sup>) anti-human STAT6, phospho-specific (Tyr<sup>1022</sup>/Tyr<sup>1023</sup>) anti-human Jak1, phospho-specific (Tyr<sup>980</sup>) anti-human Jak3, anti-human SOCS-1, anti-human SOCS-3 Abs (all from Santa Cruz Biotechnology), rabbit polyclonal phospho-specific (Tyr<sup>693</sup>) anti-human STAT4 Ab (Zymed Laboratories), and HRP-labeled anti-rabbit IgG (DakoCytomation) were used for Western blotting. Polyclonal rabbit anti-human STAT6, anti-human Jak1, anti-human STAT4 Abs (all from Santa Cruz Biotechnology), and rabbit anti-human  $\beta$ -actin (Sigma-Aldrich) were used for controlling the loading on Western blots. Polyclonal rabbit anti-human STAT6 Ab recommended for gel supershift studies (Santa Cruz Biotechnology) was used for EMSA supershift. Goat polyclonal anti-human IL-4 (R&D Systems), mouse monoclonal anti-human IL-4R $\alpha$ , HRP-labeled anti-goat IgG (both from DakoCytomation), streptavidin-biotin-HRP (Amersham Biosciences), and polyclonal biotin-conjugated anti-PIBF Abs (prepared in our laboratory) were used for ELISA. Mouse monoclonal anti-human IL-4R $\alpha$ , PE-labeled rat anti-mouse IgG2A and IgG2B (BD Biosciences), FITC-conjugated PIBF (prepared in our laboratory), PE-labeled mouse, anti-human CD45RA (BD Biosciences), and PE-labeled rat anti-mouse IgG1 (BD Biosciences) were used for immunofluorescence confocal microscopy. PIBF-FITC and mouse monoclonal anti-human IL-4R $\alpha$  Abs were used for determining the receptor binding of PIBF by flow cytometry.

### *Treatment of lymphocytes*

Ficoll-Paque (Pharmacia) isolated PBL from healthy volunteers were washed in RPMI 1640 medium (Invitrogen Life Technologies), treated with 1  $\mu$ g/ml PHA (Sigma-Aldrich) for 24 h, and then incubated at 37°C in 5% CO<sub>2</sub> with the following: RPMI 1640 medium; 10, 20, and 200 ng/ml and 0.5, 1, and 10  $\mu$ g/ml PIBF for  $5 \times 10^7$  cells; PIBF, progesterone (Sigma-Aldrich) or recombinant human IL-4 (R&D Systems) for 1, 10, 20, and 30 min and 24 h; recombinant human IL-4 plus recombinant human IL-12 (both from R&D Systems), or rIL-4 plus rIL-12 plus monoclonal anti-IL-4R $\alpha$  Ab, or rIL-4 plus monoclonal anti-IL-4R $\alpha$  Ab; PIBF plus polyclonal anti-PIBF Ab, or PIBF plus monoclonal anti-IL-4R $\alpha$  Ab, or PIBF plus rIL-12 plus monoclonal anti-IL-4R $\alpha$  Ab, or PIBF plus rIL-12 plus monoclonal anti-IL-13R $\alpha$ 1 Ab; rIL-12 plus PIBF or PIBF plus rIL-12 plus recombinant human IL-4; lysate of *Escherichia coli* that had undergone the same purification procedure as the recombinant human PIBF in a concentration of 200 ng/ml; and isotype controls used with each treatment.

The cytokines, PIBF, and progesterone were used at a concentration of 200 ng/ml for  $5 \times 10^7$  cells, whereas anti-human IL-4R $\alpha$  and anti-human IL-13R $\alpha$ 1 Abs were applied in a blocking concentration (50  $\mu$ g/ml for  $5 \times 10^7$  cells). All treatments were performed for 20 min.

### *Western blotting*

Following treatment, lymphocytes were washed in PBS and then in PBS containing 1 mM sodium vanadate and 5 mM NaF. After centrifugation at 13,000 rpm for 40 s pellets were resuspended in 3 $\times$  volumes of buffer containing 20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM sodium vanadate, 1 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 0.5 mM PMSF, aprotinin (0.01  $\mu$ g/ml), leupeptin (0.025  $\mu$ g/ml), 400 mM NaCl, and 20% glycerol. Samples were frozen and thawed three times and kept on ice for a further 20 min. After centrifugation at 15,000 rpm at 4°C for 30 min, supernatants were collected, and the protein content of the extracts was determined. The 25  $\mu$ g of total protein was separated on 7.5 or 12% SDS-PAGE and transferred to a Hybond ECL membrane (Amersham Biosciences) overnight at 54 mA. The blots were blocked with 3% nonfat dry milk in TBS-Tween solution for 60 min, then incubated with 1/20,000 diluted phospho-specific anti-STAT6 or with 3  $\mu$ g/ml phospho-specific anti-STAT4, 1/2000 diluted phospho-specific anti-Jak1, 1/200 diluted phospho-specific anti-Jak3, or with 1/200 diluted anti-SOCS-1, or with 1/200 diluted anti-SOCS-3 Abs in TBS-Tween solution containing 3% nonfat dry milk for 1 h. For controlling the loading, 1/1000 diluted anti-STAT6, 1/1000 diluted anti-STAT4, 1/1000 diluted anti-Jak1 Abs, or 1/1000 diluted anti- $\beta$ -actin Abs was used. After six washing cycles (10 min

each) in TBS-Tween (pH 7.4), the blots were incubated at room temperature for 45 min with 1/2000 diluted HRP-labeled anti-rabbit IgG, and then washed six times (10 min each) in TBS-Tween. Ab binding was detected using an ECL kit (PerkinElmer) according to the manufacturer's instructions.

### *EMSA supershift*

Nuclear extracts were prepared as described by Xu and Cooper (23). Lymphocytes were washed twice in ice-cold PBS and resuspended in 10 volumes of buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, protease inhibitors (complete, Mini EDTA-free tablets; Boehringer Mannheim), phosphatase inhibitors (Phosphatase Inhibitor Cocktail; Sigma-Aldrich) and placed on ice for 10 min. After vigorous vortexing, nuclei were collected by centrifugation in microcentrifuge at 10,000 rpm for 10 s, resuspended in 2 volumes of buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitors, and phosphatase inhibitors and incubated on ice for 20 min. After centrifugation (10,000 rpm for 10 s) supernatants were collected and the protein content of the extracts was determined, aliquoted, and stored at -80°C. For the shift assay, STAT6 binding probes (5'-TCGACTTCCCAAGAAGACAGCA-3') and their reverse complementary pairs were incubated at 80°C for 10 min in annealing buffer containing 100 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA and allowed to cool down slowly to room temperature. Double-stranded oligonucleotides were end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase (Promega) according to the manufacturer's protocol. Binding was performed at room temperature for 30 min in 20  $\mu$ l volume, containing 300 ng of poly(deoxyinosinic-deoxycytidylic acid), 100 mM NaCl, 0.25 mM EDTA, 1 mM DTT, 20 mM HEPES (pH 7.9), and 20 fmol <sup>32</sup>P-labeled oligonucleotide probe. For supershift assays the reaction mixtures were preincubated with 1  $\mu$ l of rabbit anti-human STAT6 IgG (2  $\mu$ g/ $\mu$ l) for 1 h at 4°C before adding the <sup>32</sup>P-labeled oligonucleotide. Samples were separated on 3.5% acrylamide gel with 0.5 $\times$  Tris-borate-EDTA buffer. The gel was dried and exposed to x-ray film.

### *Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment*

A total of  $5 \times 10^6$  PHA treated lymphocytes was incubated with 2.5 U of PI-PLC (Sigma-Aldrich) in 1 ml of PBS for 30 min at 37°C in 5% CO<sub>2</sub>, and washed twice with PBS.

### *ELISA for detecting PIBF binding to IL-4R*

During an overnight incubation at 4°C, 96-well microtiter plates were coated with 1 and 0.5  $\mu$ g/ml soluble recombinant human IL-4R $\alpha$  (R&D Systems) in 50 mM carbonate buffer (pH 9.6). All further incubations were performed at 37°C. Plates were washed three times and free binding sites were blocked with PBS containing 0.05% Tween, 0.5% gelatin, and 0.1% BSA for 60 min. Logarithmic dilutions (0.01–1  $\mu$ g/ml) of PIBF or rIL-4 in PBS Tween (pH 7.4) were incubated on the plates for 60 min. The plates were washed three times and incubated with 1/1000 diluted biotin-conjugated anti-PIBF IgG or 1/1000 diluted goat anti-IL-4 for 60 min. Following three further washing cycles, biotin-conjugated anti-PIBF IgG was reacted with 1/1000 diluted streptavidin-biotin-HRP for 30 min, while anti-IL-4 Ab was reacted with 1/2000 diluted HRP-labeled anti-goat IgG for 60 min. The reaction was developed with *O*-phenylenediamine (FLUKA; Sigma-Aldrich) and stopped by adding 50  $\mu$ l of 4 M H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 490 nm.

### *Determination of receptor binding of PIBF by flow cytometry*

One million PBL from healthy volunteers were incubated with 2  $\mu$ g/ml FITC-conjugated PIBF in the presence of increasing concentrations (0–200  $\mu$ g/ml) of unlabeled PIBF or monoclonal anti-IL-4R $\alpha$  Ab for 30 min at 4°C. The cells were washed in PBS, fixed in 0.5 ml 4% PBS-formalin, and analyzed by flow cytometry, using a FACSCalibur flow cytometer equipped with a 488-nm excitation laser with the CellQuest software program (both from BD Biosciences).

### *Confocal microscopy*

One million PBL from healthy volunteers were incubated with 5  $\mu$ g of FITC-conjugated PIBF for 20 min at 37°C. A total of  $1 \times 10^5$  cells was plated on poly-L-lysine-coated slides and incubated at 37°C for further 10 min, then washed twice in PBS containing 1% FCS and fixed with freshly prepared 3% paraformaldehyde in PBS for 10 min at room temperature. After washing, the plates were incubated with 0.5  $\mu$ g of monoclonal anti-IL-4R $\alpha$  or 2  $\mu$ l of PE-labeled mouse anti-CD45RA Ab for 45 min at



room temperature. Cells were washed twice and incubated with 2  $\mu$ l of PE-labeled rat anti-mouse IgG2A and IgG2B or PE-labeled rat anti-mouse IgG1 for 30 min at room temperature. Cells were washed twice and the slides were mounted with DABCO (Sigma-Aldrich). To control the specificity of capping formation, all steps were also performed at 4°C. The slides were analyzed with a Bio-Rad confocal microscope with  $\times 100$  objective, using laser excitation at 473 nm with filters  $580 \pm 16$  nm for PE and  $522 \pm 17.5$  nm for FITC. Images were analyzed using the Adobe Photoshop 7.0 software.

### RNA interference

Oligonucleotides were hand-designed to interfere exclusively with STAT6 mRNA (Ambion). As a negative control, the same nucleotides were scrambled to form a nongenic combination (controlled by basic local alignment search tool). Oligonucleotide sequences used for STAT6 RNA interference were target sequence for STAT6 AAG CAG GAA GAA CTC AAG TTT and target sequence scrambled AAA CGA GAG TGT TAT AAC TGT.

Cells were washed twice with OptiMEM (Invitrogen Life Technologies). Oligonucleotides were dissolved in RNase-free water, annealed according to the guidelines provided by the manufacturer, and incubated for 20 min at room temperature with Oligofectamine (Invitrogen Life Technologies). This mixture was added dropwise to the cells, until a concentration of 66 nM was reached. After 4 h incubation at 37°C, DMEM containing 30% FCS was added to cultures. Western blots for STAT6 expression were performed 24 h subsequent to small interfering (si)RNA transfection. STAT6 expression was markedly reduced, whereas STAT2 and STAT4 expressions were not affected (see Fig. 2A).

### Lymphocyte cultures

Intact lymphocytes, those treated with scrambled oligonucleotides and those after RNA interference were adjusted to  $10^6$  cells/ml in RPMI 1640 with 10% FCS, and cultured for 48 h at 37°C, 5% CO<sub>2</sub> in a humidified

atmosphere together with 0.2 or 10  $\mu$ g/ml PIBF or without PIBF. Supernatants were harvested for cytokine determination. Lymphocytes were lysed for Western blot analysis.

### Cytometric bead arrays

IL-10, TNF- $\alpha$ , and IFN- $\gamma$  concentrations were determined by Cytometric Bead Array (CBA; BD Biosciences or Bender MedSystems). Supernatants were incubated with labeled capture beads and detection reagent for 3 h in the dark at room temperature, and analyzed with flow cytometer (FACS-Calibur; BD Biosciences for BD arrays or alternatively Galaxy; DakoCytomation) by using the respective CBA Analysis software (BD Biosciences) and Bender MedSystems software.

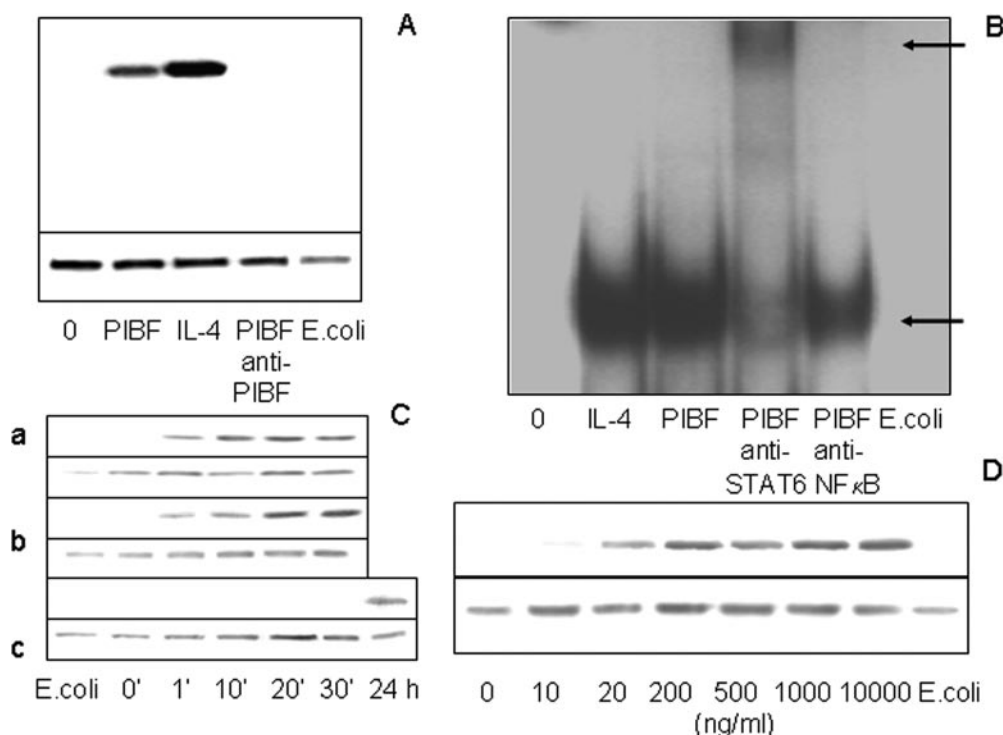
### Statistical analysis

The two-tailed Student's *t* test was used for statistical evaluation of the data. Differences were considered significant if the value for *p*  $\leq 0.05$ .

## Results

### PIBF activates STAT6

To test whether the previously described cytokine effects of PIBF were mediated by the Jak/STAT pathway, cytoplasmic fractions of PIBF-treated or untreated human PBL were separated on SDS-PAGE, blotted to nitrocellulose membranes, and reacted with anti-phospho-STAT6 Abs. Controls included the lysate of *E. coli* that had undergone the same purification procedure as the recombinant PIBF, as well as isotype controls. Similar to IL-4, PIBF induced STAT6 phosphorylation, which was inhibited by a neutralizing anti-PIBF IgG (Fig. 1A). To clarify whether PIBF induces nuclear translocation of phosphorylated STAT6 dimers, nuclear extracts prepared from PIBF-treated cells, and those from IL-4-treated as



**FIGURE 1.** PIBF activates STAT6. **A**, PIBF treatment induces STAT6 phosphorylation. Cytoplasmic fractions of untreated human PBL, and those treated for 20 min with PIBF or PIBF plus anti-PIBF IgG, were separated on SDS-PAGE, blotted to nitrocellulose membrane, and reacted with anti-phospho-STAT6 Abs. **B**, PIBF treatment induces nuclear translocation of STAT6. Nuclear extracts from untreated human PBL, and those treated for 20 min with PIBF or IL-4 were hybridized with radioactive labeled STAT6 binding specific oligonucleotide probes 5'-TCGACTTCCCAAGAACAGCA-3' and their reverse complementary pairs. The samples were separated by nondenaturing PAGE and the bands were detected by autoradiography. Supershift was performed with anti-STAT6 Ab. **C**, Time-dependence of STAT6 phosphorylation. STAT6 phosphorylation was tested on lysates of lymphocytes incubated with 200 ng/ml PIBF (**b**), IL-4 (**a**), or progesterone (**c**) for varying periods. **D**, Concentration dependence of STAT6 activation by PIBF. STAT6 phosphorylation was tested on lysates of lymphocytes incubated for 20 min with different concentrations of PIBF. Western blots were reacted with anti-phospho-STAT6 (*top*) or anti-STAT6 Ab (*bottom*).

well as untreated lymphocytes were hybridized with labeled STAT6 binding, specific oligonucleotide probes and their reverse complementary pairs. PIBF induced nuclear translocation of STAT6 in lymphocytes. The specificity of the reaction was verified with a supershift assay. This result is based on anti-STAT6 IgG-STAT6 forming high molecular mass complexes, which migrate slower than STAT6 alone.

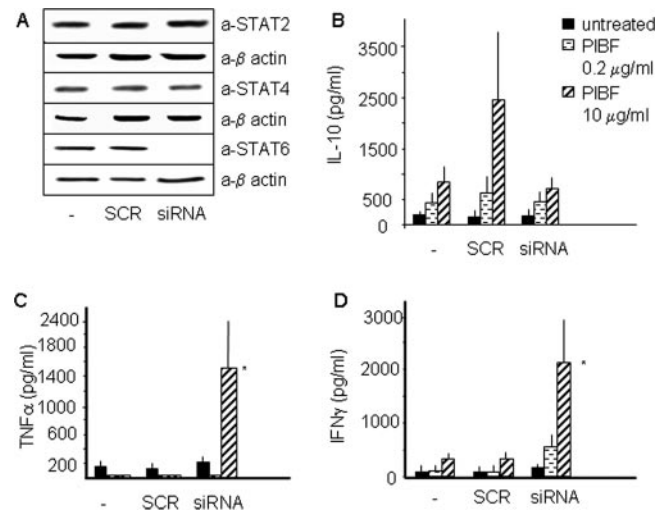
In the presence of anti-STAT6 IgG, a supershifted complex appeared in the extract of PIBF-treated lymphocytes. This band was not detectable when the irrelevant anti-NF- $\kappa$ B Ab was used (Fig. 1B).

To investigate the concentration- and time-dependence of PIBF effects, STAT6 phosphorylation was tested on lysates of lymphocytes incubated with different concentrations of PIBF or with 200 ng/ml PIBF for varying periods. Tyrosine phosphorylated STAT6 appeared as early as 1 min after addition of IL-4 (Fig. 1C, a) or PIBF (Fig. 1C, b) to the cells, whereas a 24-h continuous presence of progesterone was required for the same effect (Fig. 1C, c). PIBF in concentrations from 10 ng to 10  $\mu$ g/ml exerted a concentration-dependent effect on STAT6 phosphorylation (Fig. 1D). Our earlier data show that during normal pregnancy PIBF concentrations in urine reach 100–300 ng/ml, serum PIBF levels of the same women being usually higher. In patients with malignant tumors, PIBF levels are more variable (100–1000 ng/ml in urine). Based on these data, we chose 200 ng/ml for further treatments as a concentration relevant to in vivo biological situations.

#### STAT6-mediated signal transduction is involved in the cytokine effects of PIBF

To verify the involvement of the STAT6 pathway in the cytokine effects of PIBF, STAT6 was silenced in PBL with oligonucleotides interfering with STAT6 mRNA (siRNA). Cells treated with the same nucleotides in a scrambled, nongenomic order served as controls. The efficiency and specificity of STAT6 depletion was checked by reacting the lysates of siRNA-treated cells with anti-STAT6 or anti-STAT4 and anti-STAT2 Abs. Although the efficiency of STAT6 depletion was variable, silencing was in all cases specific for STAT6. A representative experiment is shown in Fig. 2A. STAT6 knocked down, as well as control lymphocytes, were incubated with PIBF for 48 h, and IL-10, IFN- $\gamma$ , as well as TNF- $\alpha$  concentrations of the culture medium, were determined with a cytometric bead assay. For controlling the effect of possible LPS contamination in recombinant PIBF, parallel lymphocyte samples were treated with lysates of *E. coli* that had undergone the same purification procedure as the recombinant PIBF.

Compared with the controls, the concentrations of both TNF- $\alpha$  (Fig. 2C) and IFN- $\gamma$  (Fig. 2D) were significantly increased in STAT6-deficient cells treated with 10  $\mu$ g/ml PIBF. PIBF increased IL-10 production of intact lymphocytes in a concentration-dependent manner. Similar changes were observed in cells treated with nongenomic sequence oligonucleotides and in STAT6-deficient cells. In the latter group, PIBF-induced elevation of IL-10 production was lower compared with scrambled (SCR) control; however the difference was not statistically significant (Fig. 2B). A pitfall of the RNA interference method is that according to recent findings, nonspecific effects that include both the up-regulation and suppression of nontargeted genes can be observed (24), which could account for elevated IL-10 levels in supernatants of cells treated with the nongenomic sequence. Therefore SCR control might be more appropriate as a baseline than untreated cells.



**FIGURE 2.** STAT6 is required for the cytokine effects of PIBF. The effect of PIBF on cytokine production by STAT6-deficient (siRNA) lymphocytes. Intact lymphocytes, those treated with scrambled oligonucleotides, and those after RNA interference were adjusted to  $10^6$  cells/ml in RPMI 1640 with 10% FCS and cultured for 48 h at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere together with 0.2 or 10  $\mu$ g/ml PIBF, or without PIBF. Cytokines were determined from the supernatants by cytometric bead array. Lymphocytes were lysed for Western blot analysis. Controls include intact lymphocytes (–) and those treated with nongenomic sequence oligonucleotides (SCR). *E. coli* lysate controls were included to rule out the effect of LPS contamination on cytokine production. These had no effect on IL-10 and IFN- $\gamma$  production, although they induced a slightly increased TNF- $\alpha$  production. The data shown are corrected for *E. coli* lysate control. Data are the mean  $\pm$  SEM of nine individual experiments. Significantly different (\*,  $p < 0.05$ ) values from controls are shown. A, The efficiency and specificity of STAT6 depletion by siRNA. Western blots of lysates from siRNA-treated cells were reacted with anti-STAT6, anti-STAT4, or anti-STAT2. A representative experiment is shown. B, The effect of PIBF on IL-10 production. C, The effect of PIBF on TNF- $\alpha$  production. D, The effect of PIBF on IFN- $\gamma$  production.

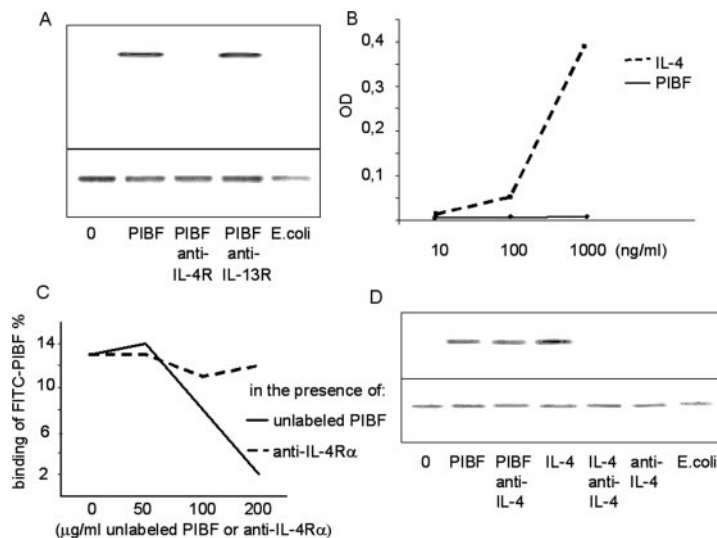
#### PIBF-induced activation of the STAT6 pathway is mediated by IL-4R

STAT6 signaling is initiated by the engagement of IL-4R. Assuming that PIBF might be a ligand of IL-4R, the STAT6 activating effect of PIBF was tested in lymphocytes pretreated with blocking concentrations of anti-IL-4R or anti-IL-13R Ab. The blocking of the IL-13R had no effect, but the use of a blocking anti-IL-4R mAb counteracted PIBF-induced STAT6 activation (Fig. 3A). This was, however, not due to the inhibition of the binding of PIBF to IL-4R because we could not demonstrate PIBF binding to IL-4R by ELISA (Fig. 3B), nor did anti-IL-4R treatment prevent PIBF binding to its own receptor (Fig. 3C). STAT6 phosphorylation by PIBF was not affected by simultaneous anti-IL-4 treatment (Fig. 3D).

PIBF induces phosphorylation of the IL-4R $\alpha$ -associated Jak1 (Fig. 4A), but not that of the  $\gamma$ -chain-associated Jak3 (Fig. 4B), and the former effect is inhibited by anti-IL-4R Ab (Fig. 4A).

Both PIBF and IL-4 treatment resulted in activation of SOCS-3, and blocking of IL-4R abolished the effect (Fig. 5A). IL-12-induced SOCS-1 activation was inhibited by PIBF, and the latter effect was abolished by blocking IL-4R (Fig. 5B). SOCS-3 inhibits IL-12 signaling, which explains the finding that IL-12-induced STAT4 phosphorylation disappeared after PIBF treatment in the absence, but not in the presence of anti-IL-4R $\alpha$  Ab (Fig. 5C). Treatment of the cells with anti-IL-12 Ab inhibited STAT4 phosphorylation, suggesting that the freshly isolated PBL were under IL-12 effect (Fig. 5D).

**FIGURE 3.** PIBF-induced STAT6 activation is mediated by IL-4R. *A*, The effect of IL-4R block on PIBF-induced STAT6 phosphorylation. Lysates of untreated human lymphocytes, and those treated for 20 min with PIBF, PIBF and anti-IL-4R $\alpha$ , or PIBF and anti-IL-13R Ab, are reacted with anti-phospho-STAT6 (*top*) or anti-STAT6 (*bottom*) Ab. *B*, Binding PIBF to IL-4R ELISA plates coated with IL-4R $\alpha$  were reacted with increasing concentrations of PIBF or IL-4, and subsequently with anti-PIBF and anti-IL-4 Abs, respectively. A representative experiment is shown. *C*, The blocking of IL-4R does not inhibit PIBF binding to its receptor. Lymphocytes were incubated with 2  $\mu$ g/ml FITC-labeled PIBF in the presence of increasing concentrations of unlabeled PIBF or anti-IL-4R Ab. The percentage of FITC PIBF binding cells was determined by flow cytometry. A representative experiment is shown. *D*, The effect of anti-IL-4 does not affect PIBF-induced STAT6 phosphorylation. Lysates of untreated human lymphocytes, and those treated for 20 min with PIBF, PIBF and anti-IL-4, IL-4, IL-4 plus anti-IL-4, or anti-IL-4 alone are reacted with anti-phospho-STAT6 (*top*) or anti-STAT6 (*bottom*) Ab.



#### Functional association of PIBF receptor and IL-4R with PIBF receptor as a GPI-anchored protein

Our data suggest that the PIBF receptor might form a heterodimer with IL-4R $\alpha$  to activate the STAT6 pathway. Confocal microscopy analysis of PE anti-IL-4R $\alpha$  and FITC PIBF-labeled cells revealed a cocapping of the two receptors, when PIBF labeling was done at 37°C (Fig. 6A, *top panel*), whereas PE anti-CD45 and FITC-PIBF did not colocalize (data not shown). Six cocapping experiments on PBL from different healthy individuals under the same conditions gave similar results. Typically cocapping was observed on 18% of the cells, and 1.7% of lymphocytes reacted with IL-4R-PE only, whereas the ratio of single FITC PIBF-positive cells was negligible.

No capping was seen in cells incubated at 4°C with the Abs (Fig. 6A, *bottom panel*).

Because IL-4R $\alpha$  was a requirement for PIBF signaling, we tested whether PIBF receptor was a GPI-anchored protein. Digesting the putative anchoring region with PI-PLC resulted in a loss of STAT6 activation by PIBF, whereas the same effect of IL-4 remained unaltered (Fig. 6B).

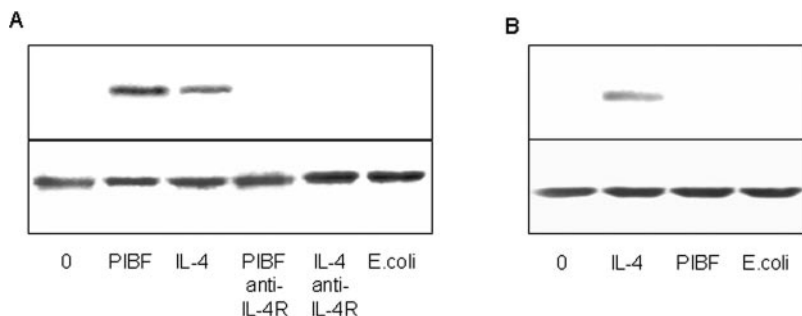
#### Discussion

In this study we suggest the existence of a new type of IL-4R, in which the  $\alpha$ -chain of the IL-4R is complemented by the GPI-anchored PIBF receptor. The treatment of human PBL with low concentrations of PIBF results in immediate phosphorylation and nuclear translocation of STAT6. STAT6 activation is dependent on ligation of IL-4R (21). This implies that for activating STAT6, PIBF needs to interact with IL-4R. Indeed, the STAT6 activating

effect of PIBF was lost after blocking the IL-4R  $\alpha$ -chain by a specific Ab. In contrast, we could not demonstrate a direct binding of PIBF to IL-4R $\alpha$ , nor could we prevent PIBF from binding to its own receptor by anti-IL-4R treatment. The possibility that the PIBF Ab binds to a region within PIBF that is required for interaction with the IL-4R, thus competing for the same site on PIBF, can be ruled out because in the ELISA we used a polyclonal anti-PIBF IgG, which recognizes multiple epitopes on the PIBF molecule. Furthermore, the assay performed in a reverse order (solid phase bound PIBF reacted with IL-4R $\alpha$ , revealed with anti-IL-4R $\alpha$ ) gave similarly negative results. Previously we showed (6) that supernatants from spleen cells activated in the presence of PIBF produce significantly more IL-4 than those in the absence of PIBF. The possible scenario in which instead of PIBF, PIBF-induced IL-4 would bind to the IL-4R and phosphorylate STAT6, can also be excluded. As short as a 1 min incubation with PIBF is sufficient to induce the phosphorylation of STAT6. This is too short an interval for gene induction plus the synthesis of IL-4. Another argument against this concept is that treatment of the cells with anti-IL-4 Abs did not inhibit, nor even reduce the effect of PIBF on STAT6 phosphorylation.

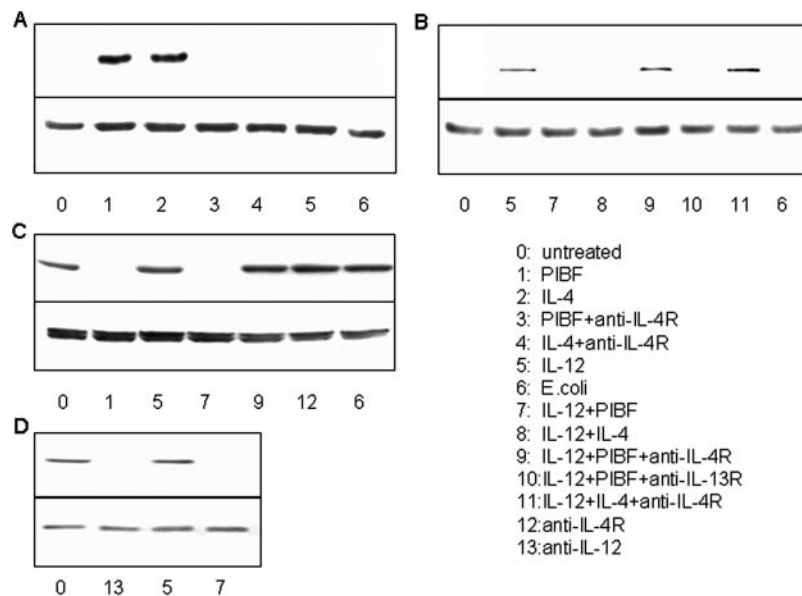
The blocking of the IL-13R (which consists of an IL-13 binding IL-13R  $\alpha$ -chain (25, 26) and IL-4R $\alpha$ ) (27) does not exert a similar effect, suggesting that the IL-4R  $\alpha$ -chain is needed for the STAT6-inducing effect of PIBF.

Because PIBF does not directly bind to IL-4R, but STAT6 phosphorylation is inhibited by anti-IL-4R Ab, we investigated the effect of IL-4R block on Jak phosphorylation in PIBF-treated cells.



**FIGURE 4.** PIBF phosphorylates Jak1, but not Jak3.

*A*, The effect of PIBF treatment and IL-4R blocking on phosphorylation of Jak1. Lysates of untreated human lymphocytes, and those treated for 20 min with PIBF, IL-4, or PIBF and anti-IL-4R $\alpha$  Ab cells, are reacted with anti-phospho-Jak1 (*top*) or anti-Jak1 (*bottom*) Ab. *B*, The effect of PIBF treatment on phosphorylation of Jak3. Lysates of untreated human lymphocytes, and those treated for 20 min with IL-4 or PIBF, are reacted with anti-phospho-Jak3 (*top*) or anti- $\beta$ -actin (*bottom*) Ab.



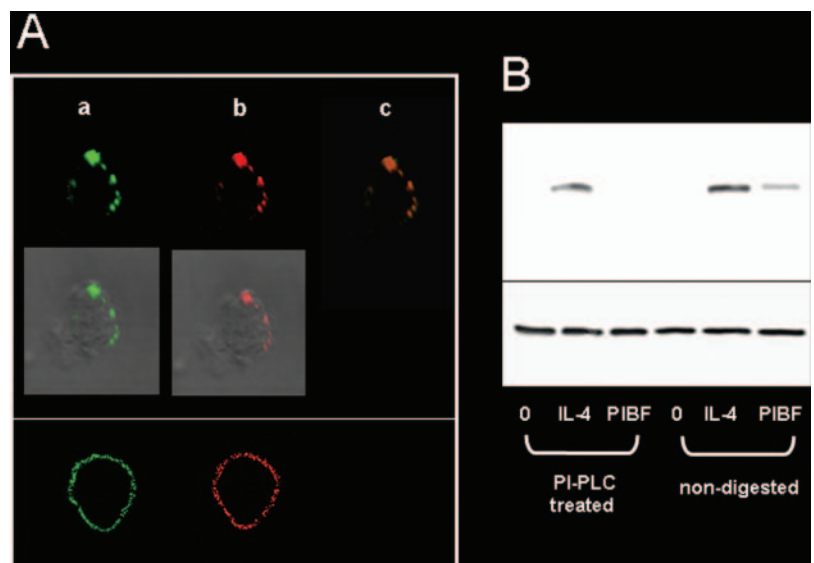
**FIGURE 5.** PIBF induces SOCS-3 and inhibits SOCS-1 induction as well as STAT4 phosphorylation via IL-4R $\alpha$ . *A*, The effect of PIBF and anti-IL-4R $\alpha$  Ab on SOCS-3 induction. Lysates of untreated human lymphocytes, and those treated for 20 min with PIBF, IL-4, PIBF and anti-IL-4R $\alpha$ , IL-4 and anti-IL-4R $\alpha$ , or IL-12, are reacted with anti-SOCS-3 (*top*) or anti- $\beta$ -actin (*bottom*) Ab. *B*, The effect of PIBF and anti-IL-4R $\alpha$  Ab on IL-12-induced SOCS-1 induction. Lysates of untreated human lymphocytes, and those treated for 20 min with IL-12, IL-12 and PIBF, IL-12 and IL-4, IL-12 and PIBF and anti-IL-4R $\alpha$ , IL-12 and PIBF and anti-IL-13R, or IL-12 and IL-4 and anti-IL-4R $\alpha$  Ab are reacted with anti-SOCS-1 (*top*) or anti- $\beta$ -actin (*bottom*) Ab. *C*, The effect of PIBF and anti-IL-4R $\alpha$  Ab on STAT4 phosphorylation. Lysates of untreated human lymphocytes, and those treated for 20 min with PIBF, IL-12, IL-12 and PIBF, IL-12 and PIBF and anti-IL-4R $\alpha$ , or anti-IL-4R $\alpha$  Ab, are reacted with anti-phospho-STAT4 (*top*) or anti-STAT4 (*bottom*) Ab. *D*, The effect of anti-IL-12 Ab treatment on STAT4 phosphorylation in freshly isolated PBL. Lysates of untreated human PBL, and those treated for 20 min with anti-IL-12 or IL-12, are reacted with anti-phospho-STAT4 (*top*) or anti-STAT4 (*bottom*) Ab.

PIBF-induced Jak1 phosphorylation was inhibited by anti-IL-4R Ab treatment, whereas the blocking of IL-13R had no effect.

STAT4 was phosphorylated in untreated cells, and this did not change upon addition of IL-12. In this study we used freshly isolated lymphocytes from healthy individuals, which produce IL-12. The treatment of the cells with anti-IL-12 Ab abolished STAT4 phosphorylation, thus phosphorylation of STAT4 in untreated lymphocytes could be due to IL-12 already present in the system. The STAT4 activating effect of IL-12 was counteracted by PIBF in the absence, but not in the presence of anti-IL-4R Ab. This implies that effects of PIBF (both the positive ones on STAT6 and the negative ones on STAT4) are dependent on the IL-4R  $\alpha$ -chain.

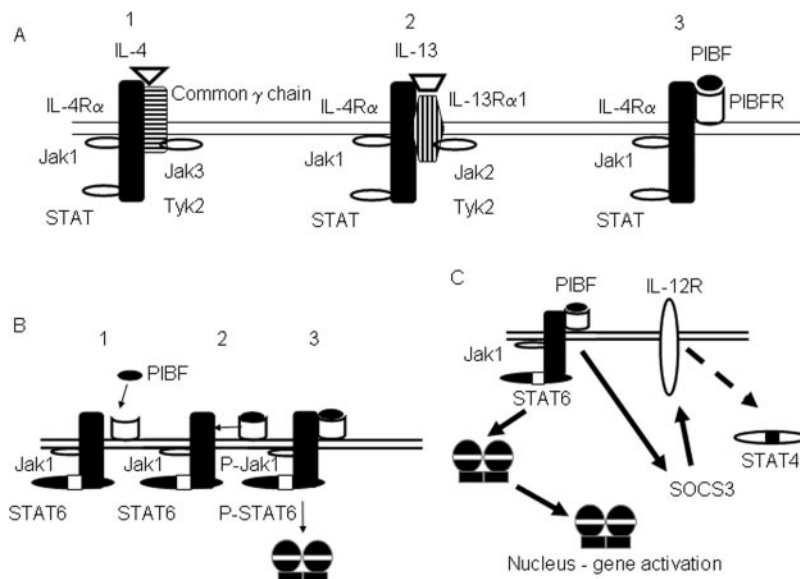
Ligand binding of IL-4R leads to phosphorylation of Jak1. Jak then phosphorylates STATs, which move into the nucleus and activate the transcription of, among others, genes coding for the SOCS proteins. IL-12/STAT4 and IL-4/STAT6 pathways are under negative feedback regulation by SOCS proteins. SOCS-3 is induced by IL-4 signal, then it binds to IL-12R and inhibits IL-12 signaling (28). Thus, IL-12-induced STAT4 activation is inhibited in Th2 cells that express high levels of SOCS-3, whereas IL-4/STAT6 signaling is constitutively activated in Th2 cells, but not in Th1 cells, with high SOCS-1 expression (16). In our hands, similar to IL-4, PIBF treatment induced SOCS-3 activation, whereas the IL-12-induced SOCS-1 expression disappeared after PIBF or IL-4

**FIGURE 6.** The PIBF receptor is a GPI-anchored protein and forms a heterodimer with IL-4R $\alpha$ . *A*, Colocalization at 4°C (*bottom*) and cocapping at 37°C (*top*) of IL-4R $\alpha$  and PIBF receptor. View of FITC PIBF (*a*), PE anti-IL-4R $\alpha$  (*b*), and merged (*c*) are presented. For capping (*top*), lymphocytes were incubated with FITC-conjugated PIBF for 30 min at 37°C, fixed and reacted with monoclonal anti-IL-4R $\alpha$  for 45 min at room temperature, followed by incubation with PE-labeled rat anti-mouse IgG2A and IgG2B or PE-labeled rat anti-mouse IgG1 for 30 min at room temperature. Fluorescence and transmission views are merged to show the cellular localization of the receptors. *B*, PI-PLC treatment results in a loss of STAT6 activation by PIBF. Cell lysates of PI-PLC digested PIBF, IL-4-treated and control untreated (marked as 0) as well as nondigested cells are reacted with anti-phospho-STAT6 (*top*) or anti-STAT6 (*bottom*) Ab.





**FIGURE 7.** PIBF acts on the cytokine balance via a novel type of IL-4R. **A**, In addition to the classical IL-4R, in which IL-4R  $\alpha$ -chain dimerizes with the common  $\gamma$ -chain (1) or the  $\alpha$ -chain (2) of the IL-13R, IL-4R  $\alpha$ -chain forms a heterocomplex with the PIBF receptor (3). **B**, Upon ligation, the PIBF receptor associates with IL-4R $\alpha$ . **C**, PIBF binding to the PIBF receptor-IL-4R $\alpha$  complex results in Jak1 phosphorylation, which in turn activates STAT6. STAT6 dimers move to the nucleus, where they activate a number of genes. PIBF-induced SOCS-3 binds to IL-12R and inhibits STAT4 phosphorylation.



treatment. All of the described effects were abolished by the IL-4R block.

Based on the currently available data, STAT6 signaling is initiated by IL-4 or IL-13 binding to IL-4R (29). Although we could not demonstrate direct binding of PIBF to IL-4R $\alpha$ , blocking of the latter suspended all IL-4R-dependent effects of PIBF. Furthermore, with confocal microscopy we demonstrated not only colocalization, but under appropriate conditions, also cocapping of IL-4R $\alpha$  and the PIBF receptor. Therefore the hypothesis was put forward that, following ligation, the PIBF receptor might either form a heterodimer with the  $\alpha$ -chain of the IL-4R, or the engaged PIBF receptor associates with the complete IL-4R, allowing PIBF to activate the STAT6 pathway. To date because of the lack of a PIBF receptor-specific Ab, coprecipitation of the two receptors has not been performed. However, the finding that PIBF treatment induces the phosphorylation of Jak1 (associated with the IL-4R  $\alpha$ -chain), but not that of Jak3, which is associated with the  $\alpha$ -chain, provides strong indirect evidence for the former and against the latter concept.

Our findings raise the question, why IL-4R $\alpha$  is needed for PIBF signaling. A plausible explanation would be that the PIBF receptor itself does not possess an intracellular domain; therefore it uses that of IL-4R $\alpha$ . Several proteins are anchored to membranes via a posttranslational lipid modification, the GPI anchor. Ligation of these proteins by Abs results in signal transduction, despite the fact that these molecules have no transmembrane or intracellular domains. Their signaling capacity is due to the association of these molecules with putative transmembrane proteins that can signal via conventional mechanisms (30).

Testing the hypothesis that the PIBF receptor was a GPI-anchored protein, we digested the anchoring region with PI-PLC. After this treatment, IL-4 was still able to activate STAT6, but PIBF failed to do so, suggesting that a GPI-anchored protein was involved in PIBF signaling. GPI deficiency causes female infertility in mice (31), but to date the protein needed for maintaining pregnancy has not been identified. PIBF deficiency ablates murine pregnancy (5), suggesting that the PIBF receptor might be considered a candidate. These findings would explain not only the pregnancy protective (5), but also the cytokine-promoting (6) and possible tumor-promoting (2) effects of PIBF.

The concept that both IL-4R $\alpha$  and PIBF receptor are required for PIBF signaling is supported by the following: 1) anti-IL-4R $\alpha$

does not prevent binding of PIBF to its receptor, suggesting that PIBF receptor and IL-4R $\alpha$  are separate entities; 2) digesting the GPI anchor abolishes PIBF-driven signaling, thus a GPI-anchored protein is required for PIBF signaling; and 3) anti-IL-4R $\alpha$  Ab inhibits PIBF-induced STAT6 phosphorylation in intact cells showing that PIBF cannot signal via its own receptor without the involvement of IL-4R $\alpha$ . Taken together, the GPI-anchored PIBF receptor is required, but not sufficient, for PIBF signaling.

Our data suggest the existence of a novel IL-4R (Fig. 7A) in which upon ligation, the PIBF receptor combines with IL-4R $\alpha$  (Fig. 7B) and induces Jak1 phosphorylation, which in turn, activates STAT6. In STAT6-deficient cells, both the Th2 cytokine-inducing and Th1 cytokine-inhibiting effects of PIBF were reduced. PIBF-induced SOCS-3, through binding to the IL-12R, inhibits STAT4 phosphorylation (Fig. 7C) and Th1 responses.

## Disclosures

The authors have no financial conflict of interest.

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